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219. THE NUTRITIVE VALUE OF PROTEINS FOR MILK PRODUCTION

V. THE EFFECT OF HIGH TEMPERATURE AND OF SEASON ON THE NUTRITIVE VALUE OF GRASS PROTEINS, THE SUPPLEMENTARY EFFECT OF THE MAINTENANCE RATION ON THE PRODUCTION RATION, AND THE EFFECT OF FEEDING A HIGH-PROTEIN RATION

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(With 11 Figures)

1. MILK YIELD AND TOTAL NITROGEN

(*a*) *The effect of high temperature of drying*

IN a previous paper(1) it was shown that the drying of blood at a high temperature had a marked detrimental effect on the nutritive value of the protein for milk production, resulting in a relatively poor utilization of the protein. This is in line with the work of other investigators(2–5) who have found a similar reduction in biological value as a result of excessive heat treatment of food proteins. In the present study an attempt has been made to ascertain whether high-temperature treatment would effect a similar reduction in the nutritive value of the protein of young spring grass. In addition experiments were carried out in order to investigate further the observation made in the previous paper that the biological value of the proteins of autumn grass was inferior to those of spring grass.

Experimental methods.

(i) *Animals.* Six Ayrshire cows were used, each animal being from 4 to 6 weeks calved. The weights of the cows varied from 950 to 1100 lb.

(ii) *Rations.* Throughout the experiment each cow received a maintenance ration of straw, oats and beet pulp, at the rate of 6 lb. starch equivalent and 0·6 lb. protein equivalent per 1000 lb. live weight. The production rations were fed at the rate of 2·5 lb. starch equivalent and 0·44 lb. protein equivalent per initial 10 lb. milk. The concentrates under investigation were fed in the production ration in quantities which supplied equivalent amounts of nitrogen to the ration, the rations being balanced with oats and maize. The actual rations fed are shown in Table I.

The grass fed was dried on a P. and M. grass drier. To obtain the scorched material the temperature of the heated air was increased from 300° C. (the

Table I. *Details of daily rations*

Cows 1, 2 and 3	Cows 4, 5 and 6
Maintenance:	Maintenance:
2·750 kg. straw	2·750 kg. straw
1·500 kg. beet pulp	1·500 kg. beet pulp
1·700 kg. oats	1·700 kg. oats
Production:	Production:
Period 1:	Period 1:
2·830 kg. oats	2·830 kg. oats
1·750 kg. maize	1·750 kg. maize
4·300 kg. spring grass*	4·300 kg. spring grass*
Period 2:	Period 2:
2·830 kg. oats	2·830 kg. oats
1·750 kg. maize	1·750 kg. maize
4·300 kg. spring grass†	4·300 kg. early autumn grass*
Period 3:	Period 3:
2·830 kg. oats	2·830 kg. oats
1·750 kg. maize	1·750 kg. maize
2·752 kg. linseed cake	4·300 kg. spring grass†
Period 4:	Period 4:
2·830 kg. oats	
1·750 kg. maize	
4·300 kg. spring grass‡	

* Dried at 300° F. † Dried at 350° F. (scorched). ‡ Dried at 350° F. (green).

normal drying temperature) to 350° C. with no alteration in the speed of travel of the belt. For the high-temperature unscorched sample the belt speed was increased, the grass being in contact with the hot air for a shorter period. The early autumn grass was cut late in September and dried at 300° C.

Results.

Milk yield. Figs. 1*a*, *b* show the daily milk yields obtained during each experimental period. The dotted lines indicate the expected lactation curve for each animal if fed throughout the experiment on an adequate diet. From the curves it is clear that high-temperature dried spring grass, whether green or scorched, caused no fall in milk yield in comparison with low-temperature dried spring grass, a food which has previously been shown to contain proteins of high biological value (1). On the other hand, grass cut in mid-autumn tended to cause a reduction in milk yield as did linseed cake, thus confirming the earlier experiments (6).

Total nitrogen of milk and urine. Figs. 2–7 show the relationship between the total output of nitrogen in the milk and urine in the experimental periods. In interpreting the results allowance must be made for the gradual increase in urinary nitrogen resulting from the normal decrease in milk yield during the lactation period, since the quantity of protein fed throughout the experiments was constant, being based on the initial milk yield.

The results show clearly that a relatively high wastage of nitrogen (as urinary nitrogen) accompanied the feeding of linseed cake, while the urinary nitrogen excretion was markedly lower with the low- and high-temperature dried spring grass than with the dried autumn grass.

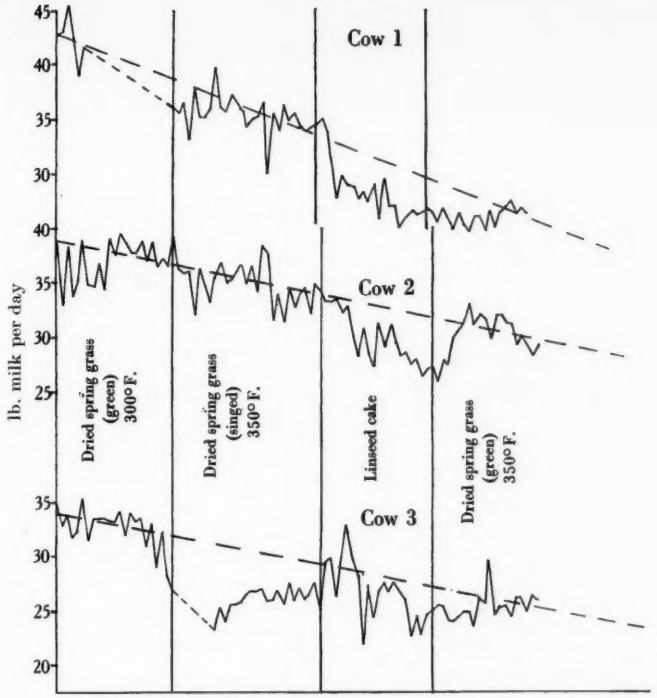
Cow 1
Cow 2
Cow 3
lb. milk per day
Dried spring grass (green) 300° F.
Dried spring grass (singed) 350° F.
Linseed cake
Dried spring grass (green) 350° F.

Fig. 1*a*.

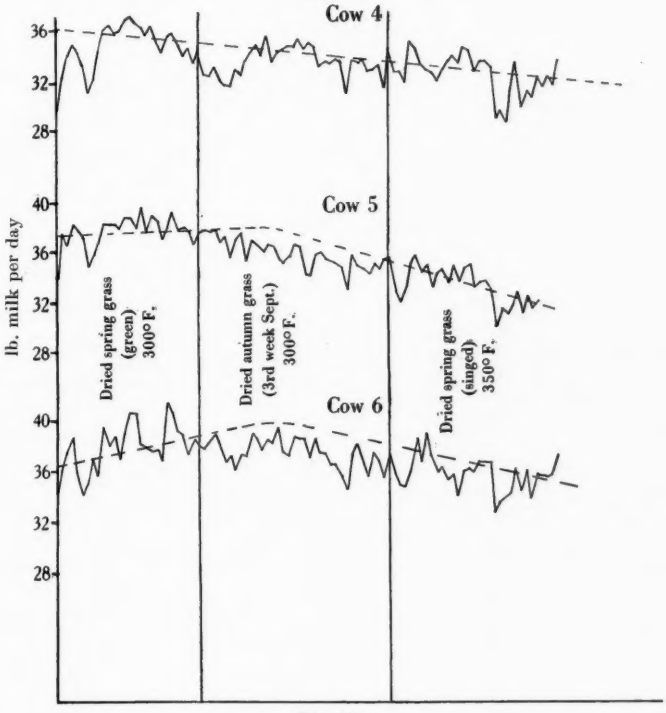
Cow 4
Cow 5
Cow 6
lb. milk per day
Dried spring grass (green) 300° F.
Dried autumn grass (3rd week Sept.) 300° F.
Dried spring grass (singed) 350° F.

Fig. 1*b*.

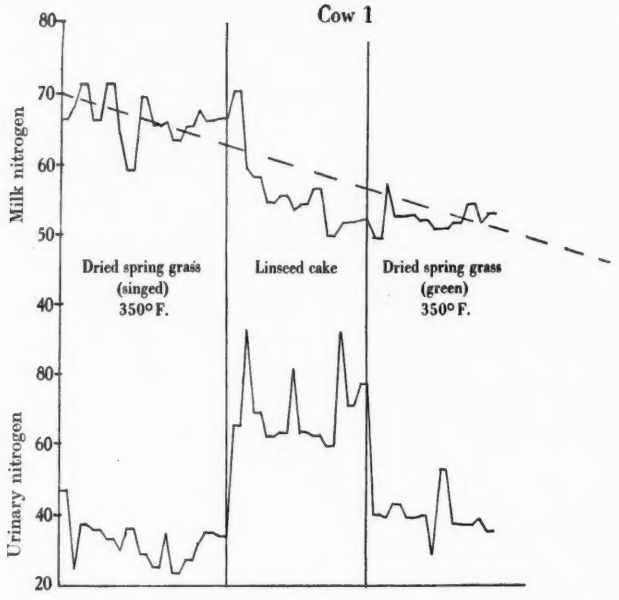
Cow 1
Milk nitrogen
80
70
60
50
40
Dried spring grass (singed) 350° F.
Linseed cake
Dried spring grass (green) 350° F.
Urinary nitrogen
80
60
40
20

Fig. 2.

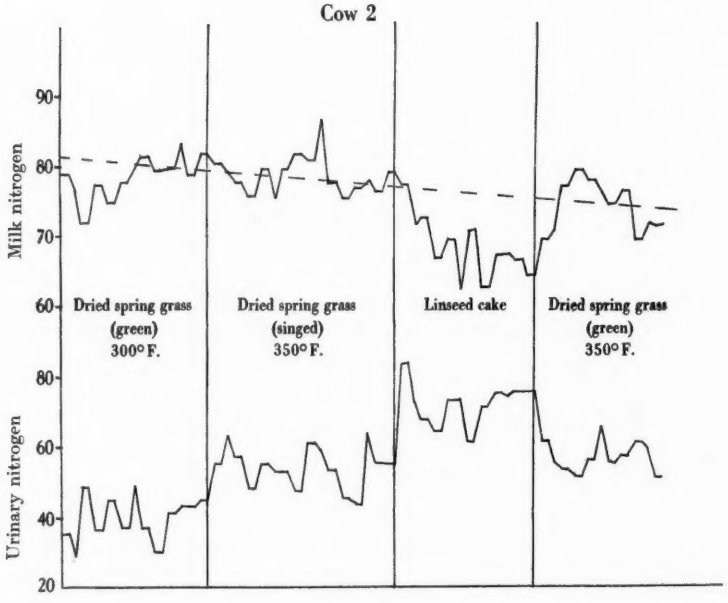
Cow 2
Milk nitrogen
90
80
70
60
Dried spring grass (green) 300° F.
Dried spring grass (singed) 350° F.
Linseed cake
Dried spring grass (green) 350° F.
Urinary nitrogen
80
60
40
20

Fig. 3.

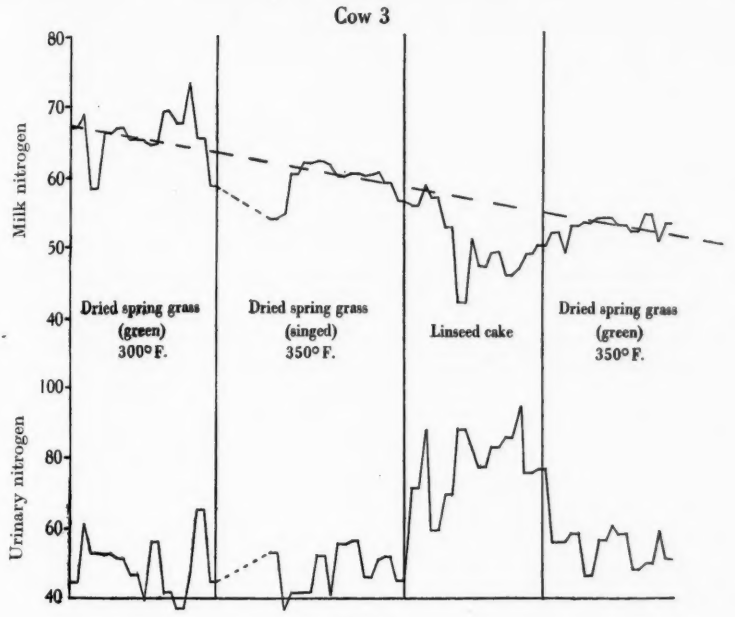
Cow 3
Milk nitrogen
80
70
60
50
40
Dried spring grass (green) 300° F.
Dried spring grass (singed) 350° F.
Linseed cake
Dried spring grass (green) 350° F.
Urinary nitrogen
100
80
60
40

Fig. 4.

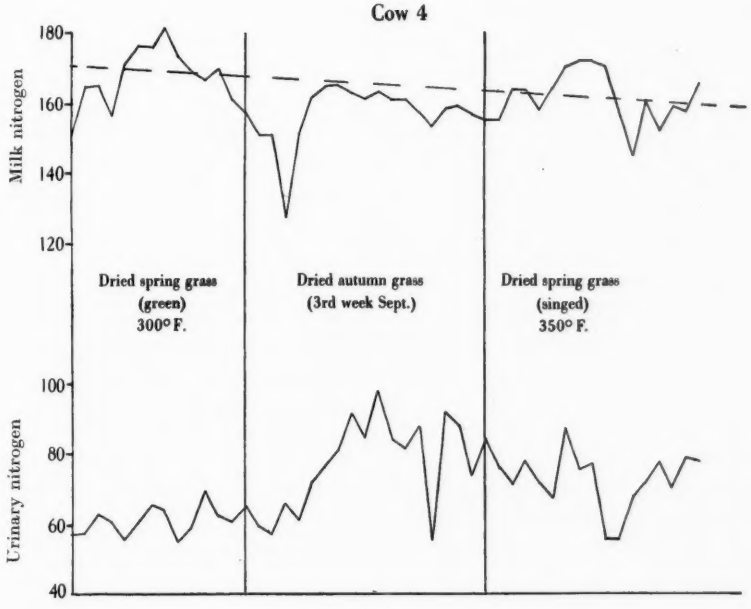
Cow 4
Milk nitrogen
180
160
140
120
Dried spring grass (green) 300° F.
Dried autumn grass (3rd week Sept.)
Dried spring grass (singed) 350° F.
Urinary nitrogen
100
80
60
40

Fig. 5.

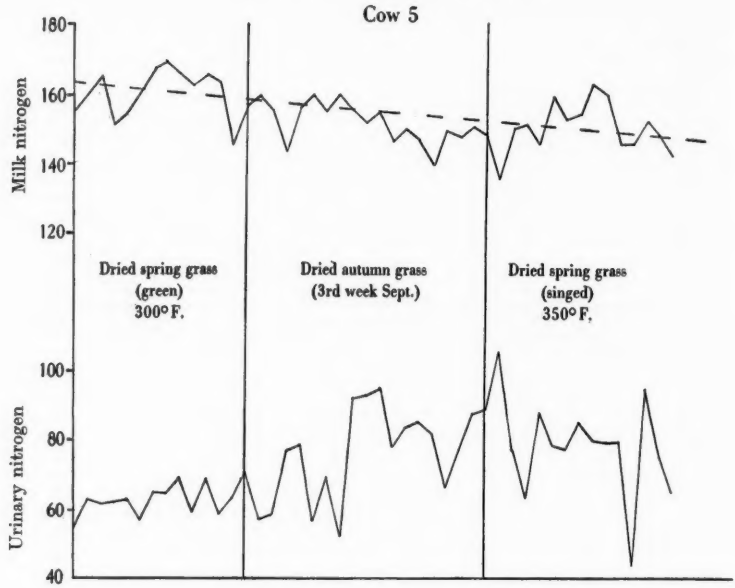
Cow 5
Milk nitrogen
180
160
140
120
Dried spring grass (green) 300° F.
Dried autumn grass (3rd week Sept.)
Dried spring grass (singed) 350° F.
Urinary nitrogen
100
80
60
40

Fig. 6.

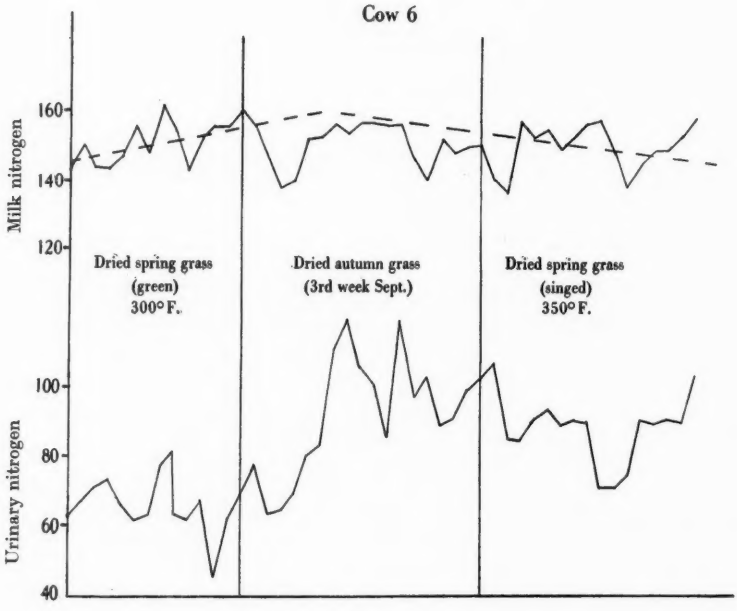
Cow 6
Milk nitrogen
160
140
120
Dried spring grass (green) 300° F.
Dried autumn grass (3rd week Sept.)
Dried spring grass (singed) 350° F.
Urinary nitrogen
100
80
60
40

Fig. 7.

(*b*) *The supplementary effect of the maintenance ration*

In a previous paper (1) it was shown that two proteins, each of low biological value, could supplement one another and give a food of high biological value, provided that the amino-acid deficiency in each protein was different. This confirmed the work of other investigators (7–10). In the present study an attempt has been made to ascertain whether a protein of high biological value fed in the maintenance ration can supplement a poor-quality protein in the production ration. A study has also been made of the effect of increasing the protein intake of the production ration on the yield of milk and the urinary nitrogen output. It may be noted that, whereas some investigators have recorded an increase in milk yield as a result of increasing the protein level in the diet (11), others have noted a decrease (12,13), while still others have found no change in the amount of milk produced (14–16).

Experimental methods.

(i) *Animals.* Three Ayrshire cows were used, each animal being from 4 to 6 weeks calved. The weights of the cows varied from 930 to 1050 lb.

(ii) *Rations.* To examine the possibility of the supplementary effect of the maintenance on the production ration a basal amount of oats and beet pulp was fed, the roughage being varied, either hay or straw being used. The amount of hay fed was smaller than that of straw in order to keep the nitrogen intake from the roughage constant. In the periods when hay was fed the maintenance ration supplied lysine in excess of actual maintenance requirement, whereas with straw only the required amount, 0·8 g. lysine, was fed. With the hay ration there was therefore a surplus of lysine which might be utilized for production.

The production ration was, too, varied in order to supply in certain periods 0·44 lb. protein equivalent per 10 lb. milk and in others 0·60 lb. protein equivalent. In all the periods the foods used were the same and the total production dry matter and energy were kept constant, the energy at 2·5 lb. starch equivalent per 10 lb. milk. The proportions of each food in the ration were changed in the high-protein period with (as noted above) the intention of supplying 0·60 lb. protein equivalent per 10 lb. milk. Owing, however, to the fall in milk yield when this ration was fed, it was found in practice to be supplying about 0·90 lb. protein equivalent.

The rations used are shown in Table II.

Results.

Milk yield. Fig. 8, which gives the daily milk yield, shows that certain of the rations caused a slight but definite lowering of the milk yield. The dotted lines indicate the expected lactation curve for each animal if fed throughout the experiment on an adequate diet. It will be seen that when straw replaced hay the milk yield was below the expected level. Increase in the level of

protein also caused a fall in milk production, although the lysine supplied (Table IX) was far in excess of actual requirement.

Table II. *Details of daily rations*

Period		Cows 7, 8 and 9		
1	Maintenance	4·550 kg. straw 1·700 kg. oats 1·500 kg. beet pulp		
	Production	9·00 kg. cubes A	6·00 kg.	7·20 kg.
2	Maintenance	1·360 kg. hay 1·700 kg. oats 1·500 kg. beet pulp		
	Production	As in period 1		
3	Maintenance	As in period 2		
	Production	9·00 kg. cubes B	6·00 kg.	7·20 kg.
4	Maintenance	As in period 2		
	Production	As in period 2		

	Cubes A %	Cubes B %
Earth-cut cake (38%)	19	50·0
Pollards	37	21·5
Maize meal	37	21·5
Molasses	5	5·0
Minerals	2	2·0

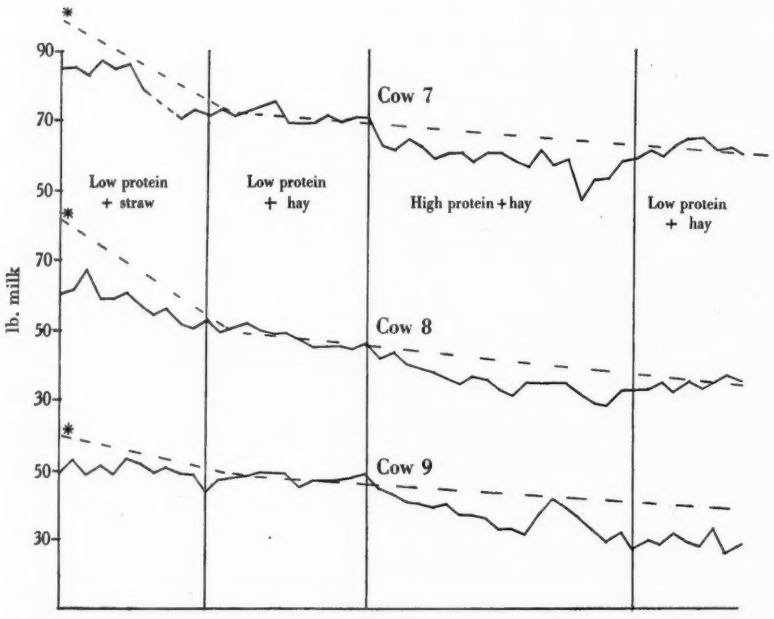

Fig. 8.

* Since the first periods were on a low protein ration, the expected lactation curves (broken lines) have been based on the initial milk yields on an adequate diet.

In this connexion it is interesting to note that the results of the high-protein diet confirm those of the previous experiments(1,6) in which a poor-quality protein, fed at the end of lactation and supplying more than sufficient lysine, could not maintain milk production at the normal level. It seems possible that an animal not only requires adequate quantities of essential amino-acids for milk production, but must have these amino-acids supplied in certain definite fixed proportions before they can be utilized in the most economic manner.

Total nitrogen of milk and urine. Figs. 9–11 show the relationship between the total output of nitrogen in the milk and urine during the experimental periods. In the low-protein periods allowance must again be made for the

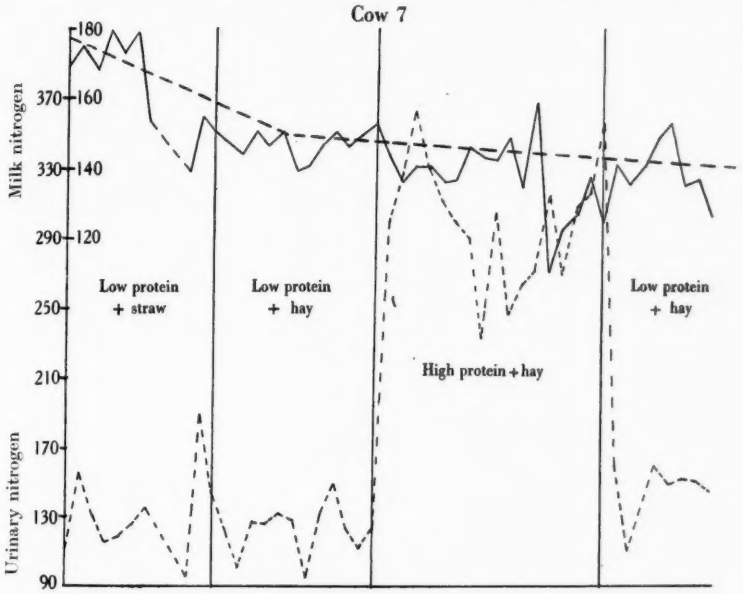

Fig. 9.

gradual increase in urinary nitrogen resulting from the normal decrease in milk yield during the lactation period.

The results show no difference in the wastage (urinary nitrogen) output in the straw and hay periods. In the high-protein period the wastage increased markedly and accounted for a large part of the excess nitrogen fed.

The percentage of nitrogen in the milk showed a marked increase in the high-protein period, falling off only slowly with the succeeding low-protein ration, as shown from the following figures:

	Period			
Cow	1	2	3	4
7	0·449	0·454	0·520	0·496
8	0·520	0·536	0·605	0·593
9	0·460	0·473	0·559	0·545

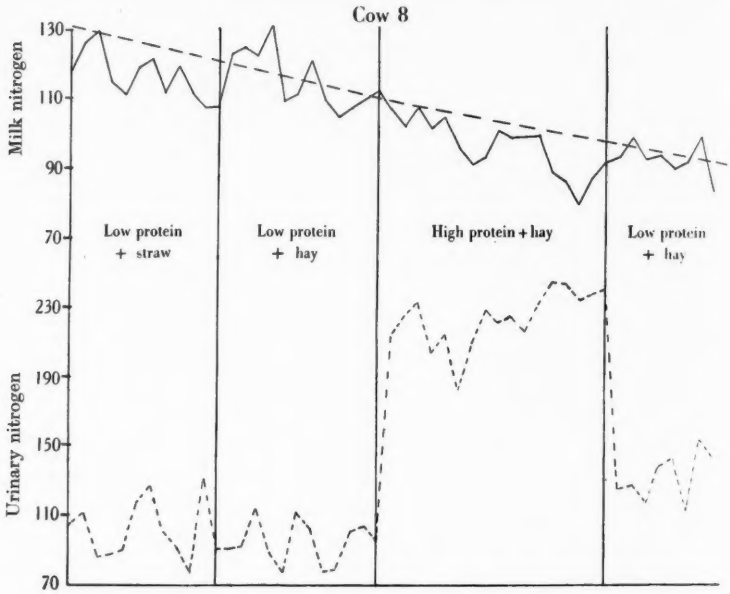
Cow 8
Milk nitrogen
130
110
90
70
Low protein + straw
Low protein + hay
High protein + hay
Low protein + hay
230
190
150
110
70
Urinary nitrogen

Fig. 10.

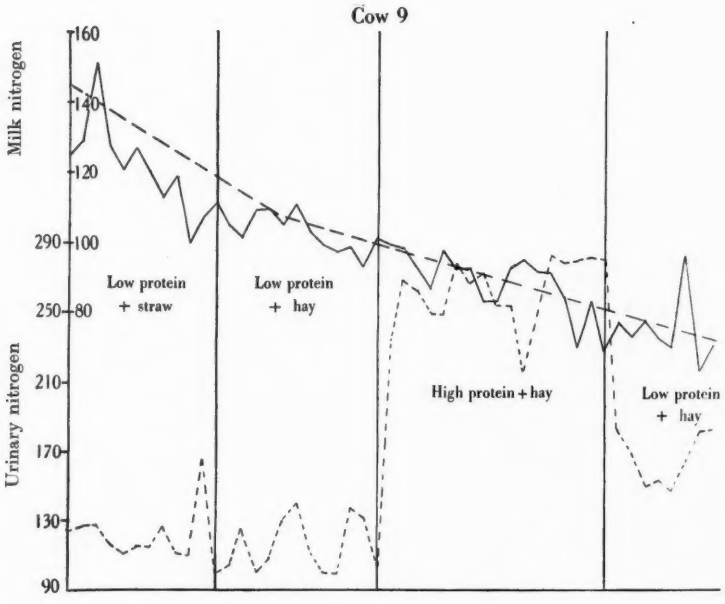
Cow 9
Milk nitrogen
160
140
120
100
80
Low protein + straw
Low protein + hay
High protein + hay
Low protein + hay
290
250
210
170
130
90
Urinary nitrogen

Fig. 11.

2. NITROGEN METABOLISM

Two-day urine and faeces samples were collected throughout each period, and analysed for total nitrogen. In addition, the urine samples were analysed for partition products. With cows 1, 2 and 3 no analyses were made of the purin bodies or hippuric acid. The analytical methods used were: total nitrogen (Kjeldahl), urea and ammonia (Folin aeration), hippuric acid (Griffith), creatinine and creatine (colorimetric), amino-acid (Sorensen formol titration), uric acid (Folin colorimetric), purin base (Kruger and Schmidt), allantoin (Ray).

Discussion of results

Nitrogen balance. The results of the nitrogen balance are shown in Table III. Fairly large differences were noted, the positive and negative balances being at times remarkably high. This was probably due to the effect of relatively short experimental periods. Over the complete experimental period the balance was positive for each animal, i.e. there was a tendency to retain part of the ingested nitrogen. With cow 7 the positive balance was exceptionally

Table III. *Nitrogen balance*

Cow	Period	Intake g.	Output g.	Daily gain or loss g.
1	1	—	—	—
	2	387·65	396·92	−4·64
	3	387·65	380·96	+3·35
	4	387·65	378·80	+4·43
2	1	440·50	444·70	−2·10
	2	440·50	450·20	−4·85
	3	440·50	426·82	+6·84
	4	440·50	443·20	−1·35
3	1	417·65	433·00	−7·68
	2	417·65	412·28	+2·69
	3	417·65	406·04	+5·81
	4	417·65	408·76	+4·45
4	1	417·65	418·66	−0·51
	2	417·65	409·99	+3·83
	3	437·65	440·37	−1·36
5	1	417·65	418·85	−0·60
	2	417·65	412·63	+2·51
	3	417·65	413·46	+2·10
6	1	427·65	420·09	+3·78
	2	427·65	428·00	−0·18
	3	437·65	433·68	+1·99
7	1	483·10	485·82	−1·36
	2	483·10	470·16	+6·47
	3	642·30	623·40	+9·45
	4	483·10	476·28	+3·41
8	1	349·10	349·64	−0·27
	2	349·10	353·52	−2·21
	3	462·00	463·80	−0·90
	4	349·10	347·93	+0·59
9	1	393·00	384·66	+4·17
	2	383·00	374·39	+4·31
	3	511·10	511·45	−0·18
	4	383·00	393·91	−0·46

high, especially in the period of high-protein feeding. This animal was fairly lean at the beginning of the experiment.

Faecal nitrogen. The present series of experiments with cows 1–6 confirm the results previously noted[1,6,17,18] that, when a deficient protein is fed, the apparent digestibility of the total ration is higher than when an adequate protein is ingested (Table IV).

Table IV. *Faecal nitrogen excretion*

Cow	Period	Faecal N (g.)	% digestibility
1	1	—	—
	2	196·00	49·4
	3	112·76	70·9
	4	195·30	49·6
2	1	198·80	54·9
	2	195·80	55·6
	3	147·02	66·6
	4	183·20	58·4
3	1	203·00	51·4
	2	200·64	52·0
	3	152·62	63·4
	4	198·80	52·4
4	1	190·50	54·4
	2	176·45	57·7
	3	208·41	52·3
5	1	195·33	53·2
	2	185·57	55·6
	3	186·54	55·3
6	1	205·39	51·9
	2	186·72	56·3
	3	199·38	54·4
7	1	189·01	61·0
	2	200·33	59·0
	3	185·80	71·1
	4	189·16	60·8
8	1	131·18	63·0
	2	143·18	59·0
	3	144·40	68·7
	4	123·17	64·6
9	1	150·90	62·0
	2	165·11	57·0
	3	160·70	68·5
	4	153·28	61·0

In the periods of low-protein intake the present experiment also confirms the previous results, a decrease in the apparent digestibility being found to accompany an improvement in the quality of the protein fed. In the high-protein period, however, the apparent digestibility increased markedly, contrary to results obtained using steers[19,20]. When compared with the results using lactating animals[1,6] a close similarity is found. When a poor-quality protein was fed at the end of lactation and supplied more than sufficient nitrogen and amino-acids for milk production, the biological value was found to be low and the faecal nitrogen excretion low, i.e. a high nitrogen digestibility. In the present instance a foodstuff of low biological value is being fed at a high level, far in excess of requirement, and a similar finding is recorded, a high digestibility of the nitrogen. It would (as noted in the previous section)

appear that not only is the total intake of lysine a controlling factor in milk production, but that the total amino-acid composition of the ingested protein plays an important part.

Urinary partition of nitrogen

(i) *Exogenous metabolism.* It has already been shown that the feeding of rations with poor-quality proteins results in an increased wastage of nitrogen as measured by the total urinary nitrogen output. Tables V and VI show that this also results in an increase in the excretion of exogenous metabolites through deamination of the excess circulating amino-acids. During these periods both total and percentage ammonia nitrogen increase, with a corresponding decrease in urea nitrogen excretion. When linseed cake was fed the total amino-acid nitrogen excretion rose but the percentage fell markedly. From a survey of all the results obtained it seems possible that there is little significance in the variations of the amino-acid nitrogen output. When excessive amounts of protein were fed (cows 7–9, period 3) there was little or no change in the amino-acid nitrogen excretion, whereas the ammonia nitrogen rose in total amount, although the percentage excretion decreased. The urea nitrogen, on the other hand, increased in total and percentage amount. These results agree with those of other investigators who studied the effect of an increased protein intake on the nitrogen metabolism of humans (21,22) and ruminants (23).

In previous publications (1,6,17,18) it has been stated that the high ammonia nitrogen excretion, during those periods in which a food of low biological value was fed, was possibly the result of an excessive deamination with the production of a mild acidosis. The results obtained when feeding an excessive amount of protein would appear to contradict this hypothesis, unless ammonia formation in the ruminant cannot exceed a definite maximum value. Further investigation on this point is necessary.

The excretion of hippuric acid nitrogen only appears to increase markedly with a large increase in urinary nitrogen output. With the grasses the low-temperature dried spring grass gives a greater excretion of hippuric acid nitrogen than the early autumn grass or the high-temperature dried material. In this instance the hippuric acid nitrogen is lowest when the total nitrogen output is high. The low excretion with cows 4, 5 and 6 in periods 2 and 3 is possibly due to a decrease in the benzoic acid content of the grass, either seasonal, as in the early autumn grass, or caused by drying at a high temperature.

Warth (24) stated that the hippuric acid nitrogen excretion determined to a certain extent the level of the urinary nitrogen output. As Csonka (25) and McCollum & Hoagland (26) have shown, however, this only occurs when benzoic acid is added to a diet deficient in glycine. In the present experiment the results show that only when the urinary nitrogen excretion is markedly increased is there any change in the hippuric acid nitrogen output.

(ii) *Creatinine and creatine.* With regard to the creatine output, large

Table V. *Urinary excretion of nitrogen (average 2-day)*

Cow	Period	Total N	Urea N	Ammonium N	Amino-acid N	Hippuric acid N	Preformed creatinine	Creatinine	Uric acid	Purin base	Allantoin
1	1	—	—	—	—	—	—	—	—	—	—
	2	61·70	24·52	12·96	4·32	—	10·62	4·84	—	—	—
	3	137·20	38·64	40·42	6·04	—	10·98	11·34	—	—	—
	4	79·40	36·04	10·16	5·08	—	12·70	12·70	—	—	—
2	1	89·60	48·00	11·38	5·56	—	9·08	4·30	—	—	—
	2	102·80	41·58	13·00	7·92	—	10·44	4·60	—	—	—
	3	143·00	33·60	34·16	3·42	—	12·70	11·06	—	—	—
	4	111·80	25·92	12·06	7·70	—	11·30	3·58	—	—	—
3	1	99·00	53·42	12·56	8·50	—	10·56	3·44	—	—	—
	2	96·04	43·12	11·22	7·28	—	10·34	2·50	—	—	—
	3	153·84	30·76	52·90	4·00	—	9·94	13·12	—	—	—
	4	105·96	50·10	12·72	7·94	—	11·30	1·70	—	—	—
4	1	61·04	26·00	8·04	3·96	6·16	7·89	1·92	1·97	4·81	17·71
	2	76·91	12·41	37·08	6·64	3·51	6·31	3·42	1·83	2·85	25·00
	3	72·00	32·42	18·54	6·00	2·32	5·68	2·22	1·73	5·73	21·00
5	1	63·43	28·22	6·23	2·97	9·77	8·53	0·86	1·08	4·04	18·63
	2	75·84	21·91	22·01	6·15	4·56	7·14	2·09	1·85	3·12	22·51
	3	78·60	27·92	18·96	8·08	4·36	7·12	1·47	1·42	3·66	23·14
6	1	64·60	29·05	5·33	3·74	8·86	7·72	1·60	1·11	4·10	17·74
	2	92·00	31·90	20·84	5·74	8·32	7·78	3·90	1·87	3·60	25·91
	3	88·00	28·60	23·90	7·70	5·22	8·16	1·47	1·42	3·66	23·14
7	1	132·61	34·10	62·59	13·98	1·36	11·93	8·42	3·46	6·24	42·73
	2	122·63	67·30	25·83	5·98	1·60	12·65	4·29	3·19	4·34	34·21
	3	300·60	182·96	50·14	15·86	7·45	16·35	7·94	6·10	5·33	91·50
	4	147·11	63·35	32·46	18·41	2·80	13·17	5·60	4·41	8·39	53·30
8	1	101·86	31·15	36·61	12·40	1·30	7·84	6·99	2·55	3·37	28·98
	2	94·74	43·41	21·34	6·84	1·45	8·64	4·20	2·40	2·48	28·16
	3	223·50	144·96	21·06	8·43	6·15	13·04	6·76	5·15	2·15	77·10
	4	132·21	79·63	14·46	9·44	3·79	11·10	6·46	3·84	5·81	47·27
9	1	113·11	35·21	44·05	12·04	1·49	9·22	7·52	2·40	1·84	32·33
	2	116·38	54·47	27·84	7·32	1·79	9·85	4·82	2·40	3·04	31·08
	3	262·50	149·32	50·48	17·26	5·10	12·93	7·55	5·41	3·73	77·70
	4	166·01	90·12	32·20	10·90	3·57	10·90	6·80	3·70	4·94	51·06

Table VI. *Nitrogen excretion (as percentage of total nitrogen)*

Cow	Period	Urea N	Ammonium N	Amino-acid N	Hippuric acid N	Preformed creatinine N	Creatine N	Uric acid N	Purin base N	Allantoin N
1	1	—	—	—	—	—	—	—	—	—
	2	39·8	21·0	7·0	—	6·4	2·3	—	—	—
	3	28·2	29·5	4·4	—	3·0	3·1	—	—	—
	4	45·4	12·8	6·4	—	6·0	1·5	—	—	—
2	1	53·6	12·7	6·2	—	3·8	1·8	—	—	—
	2	40·4	12·6	7·7	—	3·8	1·7	—	—	—
	3	23·5	23·9	2·4	—	3·6	2·9	—	—	—
	4	46·4	10·8	6·9	—	3·8	1·2	—	—	—
3	1	54·0	12·7	8·6	—	4·0	1·3	—	—	—
	2	44·9	11·7	7·6	—	4·0	0·9	—	—	—
	3	20·0	34·4	2·6	—	2·4	3·2	—	—	—
	4	47·3	11·2	7·5	—	4·0	0·6	—	—	—
4	1	42·6	13·2	6·5	10·1	4·8	1·2	1·1	2·6	9·7
	2	16·1	48·2	8·6	4·6	3·1	1·7	0·8	1·2	11·6
	3	45·0	25·8	8·3	3·2	3·0	1·2	0·8	2·7	10·5
5	1	44·5	9·8	4·7	15·4	5·0	0·5	0·6	2·1	9·8
	2	28·9	29·0	8·1	6·0	3·5	1·0	0·8	1·4	10·6
	3	35·5	24·1	10·3	5·5	3·4	0·7	0·6	1·6	10·5
6	1	44·9	8·2	5·8	13·7	4·5	0·9	0·6	2·1	9·2
	2	34·7	22·6	6·2	9·0	3·2	1·6	0·7	1·5	10·1
	3	32·5	27·2	8·8	5·9	3·5	1·2	0·6	1·8	9·8
7	1	25·7	47·2	10·5	1·0	3·4	2·4	0·9	1·6	11·5
	2	54·9	21·1	4·9	1·3	3·9	1·3	0·9	1·2	10·0
	3	60·8	16·7	5·3	2·5	2·0	1·0	0·7	0·6	10·9
	4	43·0	22·2	12·5	1·9	3·4	1·4	1·0	1·9	12·9
8	1	30·6	36·0	12·2	1·3	2·9	2·6	0·8	1·1	10·2
	2	45·8	22·5	7·2	1·5	3·4	1·7	0·8	0·9	10·6
	3	64·8	9·4	3·8	2·8	2·2	1·1	0·8	0·3	12·3
	4	60·2	10·9	7·1	2·9	3·1	1·8	1·0	1·5	12·8
9	1	31·1	39·0	10·7	1·3	3·1	2·5	0·7	0·5	10·2
	2	46·8	23·9	6·3	1·5	3·2	1·6	0·7	0·9	9·5
	3	56·8	19·2	6·6	1·9	1·9	1·1	0·7	0·4	10·6
	4	54·3	19·4	6·6	2·1	2·5	1·5	0·8	1·0	11·0

variations are found. A parallelism can be found between the total urinary nitrogen excretion and the creatinine output. From previous work[1,6,17,18] and from the experiments of Hutchinson & Morris[27] it was concluded that an increase in creatine excretion was the result of an increased tissue metabolism and not of an intensified deamination. Even when feeding excessive amounts of protein the creatine output is not increased. The present results do not confirm the claim of Terroine[28] that an increase in the amount of protein ingested gives an increase in the amount of creatine excreted.

Regarding the creatinine metabolism, attention must be drawn to the fact that throughout the whole series of experiments[1,6,17,18] the creatinine excretion remains constant in any one experiment, being unaffected by the quality of the protein fed. However, increasing the nitrogen ingested causes a marked increase in the creatinine elimination with no alteration in the creatinine intake.

The percentage output of both creatinine and creatine varies with the total nitrogen excretion and with the nutritive value of the proteins fed. Whereas the creatinine falls in percentage amount, the creatinine increases with a decrease in the biological value of the proteins, except when the protein intake is very high. In this instance both creatinine and creatine fall in percentage amount, confirming results previously obtained with humans[21,22] and ruminants[23].

(iii) *Purin metabolism.* It will be seen that the only marked change in the purin metabolism is found in the allantoin excretion, which increases with the urinary nitrogen output. A small increase is also found in the uric acid excretion, while the purin base remains fairly constant. As regards percentage output the purin base falls with increasing nitrogen excretion, the percentage output of uric acid and allantoin remaining constant.

Table VII shows the distribution of purin nitrogen. As the urinary nitrogen increases the purin nitrogen decreases, with a corresponding increase in the allantoin excretion. Terroine[29] has shown that with an increased protein intake the urinary allantoin increases with the urinary nitrogen. From this and the results obtained during inanition he states that allantoin can be synthesized from body protein or ingested protein. In Terroine's experiments this may possibly be the case, since it is a well-known fact that histidine may function as a precursor of the purins[30]. In the present instance, however, the increased allantoin output can probably be accounted for by an increased purin intake in the high-protein period.

(iv) *Sulphur metabolism.* Table VIII shows the results of the sulphur and nitrogen determinations in the urine. As previously noted[1,6,17,18] the S : N ratio of the urine is greater than that of the ration fed, due to the very low ratio in the milk. Little evidence as to the nature of the katabolism occurring in the body can be obtained from the S : N ratio of the urine. If, however, during any feeding period an excess katabolism occurs, the nature and origin may be determined from a study of the excess nitrogen and sulphur excreted.

Table VII. *Percentage distribution of purin nitrogen in urine*

Cow	Period	Uric acid	Purin base	Allantoin
4	1	8·2	19·4	72·4
	2	5·9	8·8	85·3
	3	5·7	19·3	75·0
5	1	4·8	16·8	78·4
	2	6·3	10·9	82·8
	3	4·7	12·6	82·7
6	1	5·0	17·6	77·4
	2	5·7	12·2	82·1
	3	4·9	14·8	80·3
7	1	6·4	11·4	82·2
	2	7·4	9·9	83·7
	3	5·7	4·9	90·4
	4	6·3	12·0	81·7
8	1	6·6	9·1	84·3
	2	6·5	7·3	86·2
	3	6·0	2·2	91·8
	4	6·5	9·8	83·7
9	1	6·1	4·4	89·5
	2	6·3	8·1	85·6
	3	6·0	3·4	90·6
	4	6·0	7·5	86·5

Table VIII. *Nitrogen-sulphur ratio in urine*

Cow	Period	Urinary N	Urinary S	N : S	Excess N	Excess S	N : S of excess
1	1	—	—	—	—	—	—
	2	361·70	25·86	2·4	—	—	—
	3	137·20	32·94	4·2	—	—	—
	4	79·40	27·50	2·9	—	—	—
2	1	89·60	15·88	2·8	—	—	—
	2	102·80	19·22	2·7	6·60	3·34	2·0
	3	143·00	17·58	4·1	26·70	1·70	15·7
	4	111·80	21·36	2·6	11·10	5·48	2·0
3	1	99·00	19·84	2·5	—	—	—
	2	96·04	22·81	2·1	0·00	2·97	—
	3	153·84	21·49	3·6	27·42	1·65	16·6
	4	105·96	24·06	2·2	3·48	4·17	0·8
4	1	61·04	19·39	3·2	—	—	—
	2	76·91	26·78	2·9	15·87	7·39	2·14
	3	72·00	26·36	2·7	10·96	6·97	1·57
5	1	63·43	18·71	3·4	—	—	—
	2	75·84	21·09	3·6	12·41	2·38	5·21
	3	78·60	24·25	3·2	15·17	5·54	2·74
6	1	64·60	18·85	3·4	—	—	—
	2	92·00	26·87	3·4	27·40	8·02	3·42
	3	88·00	28·89	3·1	23·40	10·04	2·34
7	1	132·61	19·54	6·8	9·98	2·10	4·8
	2	122·63	17·44	7·2	—	—	—
	3	300·60	19·65	15·3	177·97	2·21	80·5
	4	147·11	19·77	7·4	24·48	2·33	10·5
8	1	101·86	14·42	7·1	7·12	0·77	9·2
	2	94·74	13·65	6·9	—	—	—
	3	223·50	14·92	15·0	128·76	1·27	101·3
	4	132·21	17·65	7·5	37·47	4·00	9·4
9	1	113·11	15·85	7·1	0·00	1·52	—
	2	116·38	14·33	8·1	—	—	—
	3	262·50	18·13	14·5	146·12	3·80	38·4
	4	166·01	19·61	8·5	49·63	5·28	9·4

Since the ratio of S : N in body tissue (1 : 15·7) is very much lower than that of the food ingested, it should be possible to determine whether any excess excretion in the urine is due to tissue katabolism or to excessive deamination.

It will be seen that when linseed cake was fed the S : N ratio of the excess output approximated very closely that of body tissue, whereas with early autumn grass and low protein with straw the ratio was very low, due to the presence of a marked excess of sulphur but only a small excess of nitrogen in the urine. With the high-protein ration the excess nitrogen excreted is very large compared with the sulphur, giving a very high S : N ratio, a figure to be expected from the ratio of the food. On returning to the low-protein ration the excess ratio is that of the food ingested (1 : 9·5), showing a normal katabolism of ingested protein.

When the high-temperature dried grass was fed there was a large excretion of sulphur, the S : N ratio of the excess output being very low (1 : 2). Since the sulphur intake was constant in all periods, and since no trace of tissue katabolism can be found from the results of the creatine metabolism, it appears possible that the high-temperature treatment has in some way affected the nature of the sulphur compounds in the grass, with a consequent diminution in their retention. It has already been shown with ruminants that cystine, if necessary for maintenance and milk production, need only be supplied in relatively small amounts, the absolute requirement being obtained by synthesis either in the fore-stomachs or in the blood stream from certain other sulphur compounds. From the present results it appears that certain sulphur compounds cannot be utilized in ruminant nutrition. It seems, therefore, that detailed experiments are necessary to determine which sulphur compounds can be utilized, and the effect of various forms of treatment on their availability and utilization.

3. Biological values

The formula used in the calculation of the biological values has been noted in a previous paper(1). It must be emphasized that the results refer, not to pure foods, but to those foods supplemented with oats and maize. Account must also be taken of the relatively high maintenance intake and to the possible supplementary relationship between the proteins of the maintenance ration and of the production ration.

The relatively close agreement between the values obtained with the animals will be seen from Table IX. Little if any difference was found between the low- and high-temperature dried grass. The dried autumn grass, cut in September, showed a slight fall in biological value, but this was not so marked as that found with grass cut in October(1). From the fact that there was also an alteration in the urinary partition of nitrogen, it must, however, be concluded that the proteins of the early autumn grass were slightly but definitely inferior to those of spring grass. As in a previous experiment(6), linseed cake was found to be of poor quality for milk production.

Table IX. *Biological values of rations fed*

Cow	Period	Biological value	Cow	Period	Biological value	Cow	Period	Biological value
1	1	—	4	1	75	7	1	51
	2	72		2	68		2	61
	3	50		3	74		3	40
	4	75					4	68
2	1	73	5	1	73	8	1	53
	2	70		2	69		2	71
	3	54		3	71		3	48
	4	71					4	66
3	1	69	6	1	72	9	1	47
	2	65		2	66		2	55
	3	49		3	68		3	40
	4	72					4	57

The supplementary effect of the proteins of the maintenance ration is clearly shown with cows 7, 8 and 9. When hay replaced straw as roughage an increased biological value of the ingested protein was found.

When the protein level was increased a decreased biological value was found, confirming the results of other investigators (31–32).

It is interesting to compare the biological values with the lysine intake in the ration and output in the milk for cows 7–9 (Table X). In period 1, with straw as roughage, a low biological value corresponds with a total lysine intake less than that found in the milk, showing tissue katabolism. The replacement of the straw with hay (which has a higher lysine content) gives an increased biological value (periods 2 and 4). In period 3, the effect of the increased lysine intake on the biological value is completely masked by the very high protein level of the diet.

Table X. *Biological values and lysine content of ration and milk*

Cow	Ration	g. lysine fed	g. lysine in milk	Biological value
7	1	6·30	7·65	51
	2	6·82	6·94	61
	3	9·61	6·51	40
	4	6·82	6·68	68
8	1	4·50	5·66	53
	2	5·02	5·62	71
	3	6·88	4·80	48
	4	5·02	4·69	66
9	1	5·25	5·83	47
	2	5·77	5·09	55
	3	8·02	4·48	40
	4	5·77	3·93	57

It must be concluded from these results that the supplementary effect of the maintenance ration may at times be considerable and mask completely the effect of feeding a protein with a low lysine content. This must be carefully examined before any experiment is carried out to determine the lysine value of a food by a feeding trial. A second important fact is that under normal practical conditions, where hay is fed in the maintenance ration, the level of lysine intake in the production ration can be considerably reduced.

Summary

1. As regards the nutritive value of artificially dried spring grass, the protein appears to be unaffected by the temperature of drying even to the extent of scorching the grass. Early autumn dried grass has a biological value intermediate between spring and late autumn grass, while linseed cake has a very low value.

2. The supplementary effect of the maintenance on the production ration has been shown when hay was substituted for straw in the maintenance ration together with a production protein intake of poor biological value.

3. With an excess of protein in the ration most of the excess nitrogen was excreted in the urine, while both the milk yield and the biological value of the food protein fell considerably. It is possible that, together with an adequate amount of lysine in the protein ingested, a certain mixture of amino-acids is essential before maximum milk yield can be obtained.

4. A study of the nitrogen metabolism shows that high-temperature drying has no effect, whereas marked effects were noted with the early autumn grass and more especially with the linseed cake. The hippuric acid nitrogen output decreased with the high-temperature dried grass, possibly the effect of a loss on drying of benzoic acid. With the high-protein diet an increased excretion of creatinine was noted with no alteration in the creatinine intake. The purin metabolism varies with the total nitrogen excretion, the allantoin increasing markedly in the high-protein period. This is due to an increased intake of purin base.

5. The sulphur metabolism shows the results to be expected from previous publications except in the case of the high-temperature dried grass. It appears that the high-temperature treatment has in some way affected the nature of the sulphur compounds in the grass, with a consequent diminution in the retention.

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220. THE NUTRITIONAL REQUIREMENTS OF THE LACTIC ACID BACTERIA

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Many strains of lactic acid bacteria grow poorly in plain milk but do so vigorously if certain cell extracts such as autolysed yeast or tomato juice are added. This group of milk-dysgenic strains includes many of the economically important types such as *Streptococcus dextranicus* (*Str. paracitrovorus* Hammer, *Betacoccus bovis* Orla Jensen)(1), *Lactobacillus plantarum*(2) and *L. rudensis*(3). It is a matter of interest, therefore, to study the relative efficacies of different extracts for the various types of lactic acid bacteria, and to attempt to ascertain their requirements for vitamins and other growth factors.[1] A number of enrichment substances for lactic acid bacteria have been proposed in the literature(4). In the present work the following have been compared: (*a*) dextrose, maltose, sucrose and citrate (cf. (5)); (*b*) yeast and dextrose: (i) sterilized separately, (ii) sterilized together apart from the milk, (iii) sterilized together in the milk (cf. (6)); (*c*) tomato, orange, bean, alfalfa, clover, potato and carrot extracts; (*d*) beer wort and malt extract; (*e*) peptone; (*f*) autolysed yeast; and (*g*) vitamins B_1 and B_2 and co-carboxylase (diphosphate of vitamin B_1).

Altogether, seven experiments on the effect of ten plant extracts, three sugars and three vitamins or growth factors on the growth of about 100 strains of lactic acid bacteria in milk have been carried out. The data obtained are thus too numerous to be published in detail, and the tables compiled from these can only be summarized in the present paper.

The technique adopted was that previously described(7), and the exact method of preparing the extracts and their nitrogen contents are given in the appendix.

Effect of dextrose, sucrose, maltose and citrate

The results obtained in these experiments confirmed those previously published in which it was found that some types of lactic acid bacteria were markedly stimulated by the addition of dextrose to milk(5), and showed that results similar to those found with dextrose could be obtained with maltose and sucrose. It was noticeable that sucrose was greatly superior to maltose, especially for *Streptococcus* II (pathogens) and III (heterofermentative) and *Lactobacillus* I (mesothermophilic homofermentative). Citrate was without significant effect.

[1] By "growth factor" is understood any substance which in small concentrations has a marked effect on growth in a medium rich in sugar, salts and available nitrogenous compounds.

The marked efficiency of sucrose as an enrichment factor prompted the investigation of the question as to whether sucrose can be superior to dextrose in this respect. Sucrose is known to be an excellent carbon source for *Streptococcus* III, promoting slime formation (8), and although from the theoretical point of view the possibility of the more rapid fermentation of sucrose than of dextrose is unlikely, the possible presence of growth factors adsorbed on the sugar (9,10) must be considered.

An experiment in which dextrose, sucrose, peptone + dextrose, and yeast + dextrose were compared, revealed that growth with sucrose was never faster than with dextrose. There is no advantage, therefore, in using sucrose unless a higher specificity is required.

There was little difference between yeast and the peptone used in these experiments for most types; yeast was, however, superior to peptone for some strains, especially the lactobacilli of plant origin.

THE EFFECT OF (*a*) ORANGE, TOMATO AND YEAST EXTRACTS, AND BEER WORT, AND (*b*) THE STERILIZATION OF SUGARS AND GROWTH FACTORS IN THE MEDIUM

The next experiment compared orange and tomato extracts, beer wort, and yeast + dextrose sterilized: (i) separately, (ii) together apart from the medium, and (iii) together in the medium. Yeast was found to be superior to the other extracts used. For *Streptococcus* III, yeast and dextrose heated in the medium were much more effective than when otherwise sterilized. This confirms the findings of Orla Jensen (6).

Of the three remaining extracts, beer wort was in every case much superior to the other two. In fact, in many instances, it was as good as yeast + dextrose. Orange and tomato extracts did not differ greatly. In this experiment all extracts were added at the rate of 5% irrespective of nitrogen content. The analyses of these extracts and the average times of clotting are given in Table I and the appendix. In the later experiments (Tables II–V) extracts were compared on a uniform nitrogen basis (addition of 0·01 % nitrogen) and 2 or 3% dextrose added to all media.

In Table II is given the summary of an experiment in which tomato and bean extracts were compared with beer wort and yeast at identical nitrogen concentrations (0·01% added nitrogen). In addition, data for yeast at other concentrations have been included.

Of the four extracts, beer wort was the most effective activator of acid production for all the strains tested, being only inferior to 5% yeast and tomato for *Streptococcus* II. It was invariably superior to 1% yeast (equivalent nitrogen content) and was greatly superior for *Streptococcus* III and all the lactobacilli. Tomato was, on the whole, better than bean extract and was especially good for *Streptococcus* II, being more effective than all the others except 5% yeast. Bean extract was noticeably poor for certain types, particularly *Lactobacillus*

Table I (*Exp.* 4)

Group	No. of strains	Control (plain milk)	5% yeast + 1% dextrose heated separately	5% yeast + 1% dextrose heated together	5% yeast + 1% dextrose heated in medium	5% tomato juice	5% orange juice	5% beer wort
Streptococcus I	21	3·5	1·2	1·3	1·0	2·9	3·1	1·4
Streptococcus I (milk dysgenic strains)	4	45·0	1·3	1·0	1·3	33·8	45·0	6·0
Streptococcus II	12	17·4	1·3	1·4	1·5	9·1	10·3	7·9
Streptococcus III	5	37·0	4·0	4·3	2·4	32·0	38·0	6·4
Lactobacillus I	10	7·3	1·7	1·8	1·9	6·7	5·7	2·6
Lactobacillus I (milk dysgenic strains)	6	32·2	6·3	7·2	7·7	23·7	25·2	5·8
Lactobacillus II	19	23·9	2·3	1·9	2·9	21·3	17·4	2·4
Lactobacillus III	12	49·0	19·0	23·4	22·8	44·5	50·6	23·0

The figures give the average number of days required for clotting at 30 or 37° C. Strains in group I have been divided into fast-growing and slow-growing subgroups in order to emphasize the effect of the extracts on the latter.

Analysis of added constituents (results expressed as percentage present in special litmus milk media – 5% of extract added)

	Dry matter	Ash	Total N	Non-protein N	Amino N	"Dextrose"
Autolysed yeast	0·54	0·062	0·0569	0·0567	0·0184	0·057*
Beer wort	1·20	0·018	0·0090	0·0090	0·00252	0·601
Orange juice	0·44	0·015	0·0022	0·0021	0·0008	0·253
Tomato juice	0·19	0·027	0·0056	0·0054	0·0019	0·119

* Presumably due almost entirely to non-sugar-reducing substances.

Table II (*Exp.* 7). *No. of days to clot dextrose milk*

Group	No. of strains	Control	Yeast 5%	Yeast 1%	Yeast 0·2%	Tomato	Bean	Beer wort
Streptococcus I	4	16·5	5·0	7·6	10·1	8·2	12·2	3·3
,, II	2	9·5	3·7	7·0	6·0	4·2	7·2	6·0
,, III	5	70·0	46·6	65·2	67·6	38·0	65·2	17·0
Lactobacillus I	9	25·7	7·6	14·7	20·4	7·3	12·9	5·6
,, II	2	39·5	12·0	24·0	34·5	11·0	17·5	5·5
,, III	11	45·7	26·2	45·2	49·0	19·8	29·6	10·1
Average for all strains		37·8	19·4	27·3	35·8	14·8	22·4	7·9

Plant extracts added at *N* concentration equivalent to 1% yeast (0·01% N).

plantarum. Except with *Streptococcus* I the three plant extracts were significantly superior to yeast. It must be remembered, however, that addition at equivalent rates of nitrogen necessitated a greater addition of total dry matter of the plant extracts than of the yeast.

Table III (Exp. 8). *No. of days to clot dextrose milk*

Group	No. of strains	Control	Yeast	Beer wort	Malt	Alfalfa	Yeast + beer wort	Yeast, beer wort, malt and alfalfa
Streptococcus I	5	7·8	1·6	1·8	2·0	10·6	1·8	1·8
,, II	11	17·0	10·3	2·0	2·2	15·1	9·4	9·0
,, III	9	46·1	17·3	5·0	6·2	10·1	12·6	9·1
Lactobacillus I	7	19·5	6·5	4·3	3·5	5·4	6·4	5·5
,, II	2	16·0	4·0	4·0	3·5	5·5	4·0	3·5
,, III	13	58·7	44·9	18·0	17·3	41·7	46·1	28·1
Average for all strains		47·7	14·1	5·8	5·8	14·7	13·4	9·5

Table III summarizes an experiment in which yeast, beer wort, malt and alfalfa extracts were compared at equal nitrogen concentrations.

On the whole no marked differences were found between the four extracts or the two combinations employed, viz. yeast + beer wort, and all four together (equivalent total nitrogen basis as before). Beer wort and malt were somewhat superior to the others, chiefly because of their greater efficiency for *Lactobacillus* III.

The results with alfalfa are of interest in the light of the findings of Sadler *et al.* (11) that alfalfa extract had a marked stimulating action on a *Betacoccus*. Alfalfa, although as good as the other extracts for *Str. faecalis*, was very much inferior for *Str. inulinaceus* and *Str. bovis*. It was also poor for some β-haemolytic streptococci (group II). In marked contrast it was not only as good as any other for *Streptococcus* III but superior for some strains of *Str. paracitrovorus*. Variable results were obtained for *Lactobacillus* III, alfalfa being poor for those strains isolated from milk products.

The most interesting finding of the experiments summarized in Tables II and III is that whereas both cocci and rod forms in groups I and II apparently require only those substances present in autolysed yeast, both *Streptococcus* III and *Lactobacillus* III require for optimum growth substances present in certain plant extracts but not present in yeast. This conclusion is based on the fact that in these experiments the latter types grew faster when such plant extracts, e.g. malt, were incorporated in the medium than when autolysed yeast was used. The possibility that the differences found were due to differences in the degree of oxidation of certain growth factors, e.g. sulphydril compounds, may be ruled out, as some experiments were made with freshly prepared extracts and some with stock preparations. Faster growth was obtained when freshly prepared extracts were used. It has in fact been observed that stock media, such as yeast dextrose broth, which have stood for some time, may give very poor or even no growth with the more fastidious types such as *Str. cremoris*, especially if chalk is present. It may be concluded that essential growth factors

are slowly oxidized by the air and that the oxidation proceeds faster at a neutral or alkaline reaction. The data given in Tables IV and V were obtained at a later date with a yeast autolysate prepared by a different method. Whereas the earlier media were prepared with a dry packet yeast autolysed for 2 days at 55° C., the later experiments were made with a moister bag yeast autolysed for 6 days at 55° C., this time having been found to give the optimum yield of autolysate. Both types were the Distillers' Co., Ltd., baker's yeast as commonly sold in England.

It would appear, therefore, that the longer autolysis improves the growth-promoting properties of the yeast preparation as in these experiments (Tables IV and V) yeast was, with a few exceptions, superior to the other extracts for all types including group III.

The question of the effect of various times of autolysis on the efficiency of the autolysate as a source of growth factors is under investigation.

Table IV (Exp. 9). *No. of days to clot dextrose milk*

Group	No. of strains	Control	Yeast	Beer wort	Malt	Alfalfa	Clover	Potato	Carrot
Streptococcus I	1	22	2	4	4	4	4	4	4
,, III	5	30·0	3·0	7·8	5·6	5·4	4·3	5·4	5·4
Lactobacillus I	1	22	3	4	4	4	4	4	4
,, II	2	30·0	3·0	5·0	4·5	6·5	6·0	9·0	4·5
,, III	8	29·0	8·0	14·8	12·9	20·9	18·1	18·4	14·4
Average for all strains		28·6	3·0	7·1	6·2	8·2	7·3	8·2	6·5

Table V (Exp. 10). *No. of days to clot dextrose milk*

Group	No. of strains	Control	Yeast	Malt	Beer wort	Alfalfa	Clover	Carrot	Potato
Streptococcus I	1	35	1	4	2	8	30	21	2
,, II	2	35·0	8·5	19·0	12·5	35·0	35·0	35·0	21·5
,, III	5	35·0	4·6	11·4	11·2	26·2	22·6	23·4	13·6
Lactobacillus I	1	35	4	4	4	6	6	4	4
,, II	2	35·0	5·0	5·0	4·0	10·5	17·0	5·5	3·5
,, III	7	31·8	9·7	15·6	14·8	25·0	30·8	16·7	10·5
Average for all strains		33·8	5·5	9·8	8·1	13·5	23·6	17·6	9·2

Order of efficiency of plant extracts

This has been assessed by calculating the average number of days required for dextrose milk to be clotted by the strains studied in the presence of the extracts (0·01% nitrogen). The results are given in Table VI.

Table VI

	Av. no. of days required to clot dextrose milk
(1) Yeast autolysate (6 days)	4·7
(2) Malt = beer wort	7·3
(3) Potato	8·7
(4) Alfalfa	11·1
(5) Carrot	12·5
(6) Tomato	14·8
(7) Clover (*Trifolium pratense*)	16·2
(8) Yeast autolysate (2 days)	20·7
(9) Bean	22·4

It may be emphasized that in most cases strains growing slowly in plain milk have been selected and group III types preponderate. Selection of groups or of types would result in a different order of efficiency. Considering the results of all the experiments, it may be concluded that yeast autolysed for 6 days is as good as any other extract, although if shorter times of autolysis are used, malt or beer wort may be significantly superior. For routine purposes therefore, yeast autolysate may be used unless it is found not to permit satisfactory growth. A further advantage lies in the fact that the autolysed yeast can be standardized as far as it is possible to standardize a biological product.

A disadvantage of the plant extracts is their low nitrogen content which entails bulky additions to media or preliminary concentration.

GAS FORMATION

The appearance of gas bubbles in clotted milk cultures is not, of course, a reliable criterion for gas production, as gas produced previous to clotting would not be observed. Nevertheless, most of the gas formed in such cultures appears after clotting, so that bubbles may be taken as an index of fairly vigorous gas production. Of five group III streptococci studied in Exp. 9, gas was observed in five cultures with autolysed yeast, four with alfalfa and clover, two with malt and one with potato, carrot and beer wort. Of nine group III lactobacilli cultures, gas was observed in six strains with yeast, four with alfalfa, two with clover and beer wort and one with malt, potato and carrot. No gas was ever observed in the plain dextrose milk cultures. It will be seen in Table IV that alfalfa and clover were not superior to the other extracts in accelerating the growth rate. It appears, therefore, that these two materials contain substances specifically stimulating gas formation.

VITAMIN CONTENTS OF EXTRACTS

The vitamin B_1 was estimated by the fluorometric method of Jansen (12,13) and the B_2 by the method of Whitnah *et al.* (14). The results are given in Table VII, and are assessed on a common nitrogen basis.

Table VII. *Vitamin content of extracts used*

	B_1	B_2
Yeast autolysate	+++*	++++
Malt extract	+++*	++(+)
Beer wort	++*	++
Potato extract	+	(?)
Carrot extract	+	+
Alfalfa extract	+	+
Clover extract	+	+
Tomato juice	+	(?)

* Contained co-carboxylase.

For general comparison the data available in the literature have been summarized in Table VIII.

Table VIII. *Vitamin content of raw and heated animal and plant materials*

	Vitamin A[1]		Carotene[2]		Vitamin B_1[3]		Vitamin B_2[4]		Vitamin C[5]	
Milk	130–220	49–133	—	4–32	23	—	0·1–0·3	—	0·3–2·9	0·4–1·0
Yeast (dried)	—	—	110	—	300–1000	—	3	—	—	—
Malt (barley)	—	—	0	—	1400	—	0·1–0·2	—	—	—
Potato	0	0	28–56	26	30–60	30	0·01	—	11–36	4–15
Alfalfa	—	—	9000–40,000*	—	—	—	0·7	—	70–200	—
Clover	—	—	4000–6000*	—	—	—	—	—	—	—
Carrot	0	0	2000–9600	4300	60	11	0·02	—	1–31	2·5–9·5
Bean	0	0	53–450	31	40–210	—	—	—	3	—
Tomato	0	0	320–590†	—	40	33	0·06	—	13–39	7–21
Orange	—	—	300–400†	—	40	—	0·007–0·009†	—	22–89†	30–50†

The first figures (left-hand column) are for unheated materials (15) and the second (right-hand column) for heated materials (16).

[1] Int. units per 100 g. [2] μg. per 100 g. [3] Int. units per 100 g.; 1 int. unit = μg. [4] (Riboflavin) mg. per 100 g. [5] mg. per 100 g.

* Dry-weight basis. † Juice.

It will be seen that yeast contains much more B_1 and B_2 than the other materials. Malt is equal to yeast in B_1 content but inferior in B_2. Milk is much richer in B_2 than the remaining materials, apart from alfalfa, but is somewhat inferior in B_1. Since beer wort and malt were found to be equal or superior yeast for some types in group III, it follows that these organisms require factors other than B_1 and B_2, and that on a soluble nitrogen basis beer wort and malt are richer in these factors than yeast. If B_1 and B_2 were the only factors required by lactic acid bacteria, the yeast autolysate would be greatly superior to all the other extracts. Data for vitamin A, carotene, and vitamin C have been included in Table VIII. No apparent relation between these figures and growth acceleration can be observed, except that alfalfa, which may contain specific substances stimulating the growth of some group III streptococci (11) and increasing gas production by group III organisms generally, is rich in carotene and vitamin C.

EXPERIMENTS WITH VITAMINS B_1 (ANEURIN), B_2 (RIBOFLAVIN) AND CO-CARBOXYLASE (DIPHOSPHATE OF ANEURIN)

These substances were added to standard litmus milk as follows and sterilized in the medium:

	Addition μg. per 100 ml.	Normal content of cow's milk μg.
B_1	40	30–40 (17)
B_2	200	100–150 (17)
Co-carboxylase	40	—
$B_1 + B_2$	40 + 200	—
B_1 + co-carboxylase	40 + 40	—

The less stable B_1 and co-carboxylase were also sterilized separately and added aseptically to sterilized litmus milk. In both cases the effect was approximately to double the amount of vitamins in the milk.

RESULTS

Generally speaking, the addition of vitamins had no detectable effect on the growth of the test organisms. The small differences found in some cases suggested that heating of the vitamins in the medium increased the growth-promoting properties of the medium and that co-carboxylase was superior to the other vitamins and combination of vitamins used. It may, however, be concluded that normal variations in the concentration of these substances in milk would not significantly affect the rate of growth of starter organisms.

Application to the ripening of dairy products

Good flavour in cheese and butter is perhaps the most important property of these products and group III organisms are intimately concerned in its production. Thus, group III streptococci are the chief aroma-producing agents in starters used for cream ripening. The role of group III lactobacilli in cheese ripening is not yet clear. Although present in far fewer numbers than group I types, at any rate in Cheddar cheese (2), they probably contribute

an important part of the cheese flavour, and when their proliferation is unchecked serious faults may result (18). It has been observed that many strains of *Lactobacillus* III produce a strong ester-like aroma in old milk cultures (19). It will be evident that the growth factor content of the medium is important in controlling the rate of proliferation of these types. It does not follow, however, that a rich medium and correspondingly rapid growth are the best conditions for the production of the desired aroma substances. Absence of growth factors, and so a slow growth, may be more important, since it has been shown that in many cases slow growth is correlated with a large proportion of by-products to which aroma is generally ascribed (20).

Summary

1. Sucrose and maltose behaved like dextrose, only more specifically in relation to type, in acclerating the rate of growth of lactic acid bacteria in milk. Sucrose was greatly superior to maltose but was never superior to dextrose.

2. Citrate was without significant effect.

3. The sterilization of yeast and dextrose in the medium produced a medium superior to milk + separately heated yeast and dextrose for *Streptococcus* III. For other types marked differences were not observed.

4. The plant extracts used were found to fall into the following order of ability to accelerate growth: yeast autolysed for 6 days, malt = beer wort, potato, alfalfa, carrot, tomato, clover, yeast autolysed for 2 days, and bean. Malt and beer wort were superior to yeast for some group III organisms.

5. Malt extract and beer wort contain growth factors for some lactic acid bacteria that are not present, or are present only to a small extent in yeast.

6. Alfalfa and clover appear to contain substances specifically stimulating gas production for group III types, especially the streptococci.

7. Normal variations in the content of vitamins B_1 and B_2 and co-carboxylase in milk do not appear to affect the rate of growth of starter organisms.

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APPENDIX

Methods of preparation of extracts

Autolysed yeast. See ref. (5).

Beer wort. Unhopped wort kindly supplied by Messrs H. G. Simonds was used.

Malt extract. One litre of tap water was added to 100 (200) g. of malt, the temperature raised to 50–55° C. and maintained until the starch reaction with iodine became weak. The mass was then raised to 100° C. and filtered.

Potato. 500 (1000) g. of grated peeled potato were macerated in 1000 g. tap water, heated at 100° C. for 1 hr. and then filtered.

Carrot. 500 (1000) g. of grated carrot were macerated in 1000 g. tap water, heated at 100° C. for 1 hr. and filtered.

Alfalfa and clover. 750 (1500) g. of freshly plucked plant were cut into small pieces, heated in 1000 g. tap water at 100° C. for 1 hr. and filtered.

Tomato and orange juice. This was obtained by expression from the fruit.

All extracts were heated and filtered through filter paper.

Material	Nitrogen content of the extracts (%)			
Yeast autolysed 2 days	1·14	1·015	0·963	
Yeast autolysed 6 days	1·135	1·298		
Malt (20%)	0·093	0·103		
(50%)	0·198			
Beer wort	0·18	0·130	0·148	0·150
Potato (50%)	0·033			
(100%)	0·058			
Carrot (50%)	0·009			
(100%	0·018			
Alfalfa (75%)	0·065	0·080		
(150%)	0·143			
Clover (75%)	0·055			
(150%)	0·081			
Tomato juice	0·112	0·036		
Orange juice	0·044			

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221. THE EFFECT OF SERUM AND BLOOD ON THE GROWTH OF LACTIC ACID BACTERIA IN MILK

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The growth of lactic acid bacteria in milk is a problem of the greatest importance in the dairy industry. The present paper describes experiments, carried out over a period of two years, on the effect of serum and blood on the rate of growth of these bacteria in milk. There are two main aspects of the problem:

(1) *The value of serum and blood for enrichment purposes in bacteriological media.* Many media designed for the cultivation of pathogenic organisms contain serum or blood. Moreover, some species require blood or certain constituents of blood (1). Many types of lactic acid bacteria grow poorly in milk, and although addition of yeast gives excellent growth with many species, a few do not grow rapidly even in yeast dextrose litmus milk (2). It is therefore a matter of interest how far serum and blood are able to satisfy the nutritive requirements of these strains.

(2) *The economic aspects of the effect of serum and blood on lactic acid bacteria in milk.* Cases of "slow starter", "non-acid milk", "dysgenic milk" and related phenomena have frequently been described in the literature (3–8). It is rarely possible to decide the exact cause of this phenomenon, but there is some evidence that cows may sometimes give milk which, although macroscopically normal, does not permit the ordinary rapid growth of starter bacteria. Occasionally, a cow may give milk which is drastically toxic for such bacteria so that even when considerably diluted in the bulk milk, inhibition of acid production may occur (9).

Dibbern & Lembke (10), in an investigation of "slow starter", found that lactic acid bacteria grew slowly in milk deficient in casein and high in albumin and globulin and having a high *p*H value. (Such a condition would be brought about by infiltration of serum into milk.) These workers found that 80% of their samples of "slow-working" milk came from cows suffering from mastitis or abortus infection. Similar results have been obtained by Davis & McClemont (11). In practice, normal milk (i.e. the secretion of perfect healthy udders) is rarely found, as bulk milk usually contains the secretion of udders damaged by mastitis to varying extents. The average incidence of mastitis in milking cattle appears to be of the order of 40%. It is thus probable that serum, or at any rate some constituents of serum, is regularly present in milk used for making butter and cheese. Further, Peskett & Folley (12) have shown that even "normal" milk contains traces of blood-serum albumin which is serologically

different from lactalbumin. Any stimulating or inhibiting effects, therefore, may be of significance, especially in starter propagation and cheese-making.

Experiments have been made to ascertain the effect on the rate of growth, as measured by the time required to clot separated milk, of addition of serum and blood (heated and unheated) in various concentrations. For purposes of comparison milk media containing dextrose (0·1%) (since serum and blood contain small amounts of dextrose which might possibly initiate the fermentation of lactose), yeast (5%) and dextrose (1%) have been included, the latter as a standard for enrichment effects (2).

TECHNIQUE

The general technique followed was that described in earlier papers (2, 13). The serum and blood were obtained from cows free from abortus infection, tuberculosis and mastitis.

EXPERIMENTAL RESULTS

Altogether six experiments were carried out and typical results are summarized in Table I (Exps. 3 and 4) and given in Table II (Exp. 5) and Table III (Exp. 6). The experiments were made on a number of samples of milk, serum and blood. Examination of the data, especially those summarized in Table I, showed the following results:

Table I. *Average time of clotting in days of milk containing various additions*

Group	No. of strains	Sep. milk	Litmus milk	Litmus milk + 5% heated serum	Litmus milk + 5% blood (unheated)	Litmus milk + 0·1% dextrose
Streptococcus I	18	12·9	13·8	10·8	9·6	12·7
,, II	10	15·1	11·8	5·7*	14·7	15·0
,, III	5	67·8	58·8	58·8	67·8	68·6
Lactobacillus I	14	21·2	20·3	16·0	23·2	25·7
,, II	12	5·2	5·9	4·9	6·1	7·6
,, III	10	52·2	41·5	23·2*	54·1	54·1

Group	No. of strains	Litmus milk	Litmus milk + 5% heated serum	Litmus milk + 5% heated blood	Yeast-dextrose-litmus milk
Streptococcus I	24	15·7	14·3	5·8*	1·0*
,, II	11	19·4	21·9	10·5*	1·6*
,, III	9	47·7	46·6	36·9	2·6*
Lactobacillus I	19	21·7	21·4	18·2	4·8*
,, II	18	14·9	16·1	10·7	2·8*
,, III	14	44·2	42·5	34·2	9·8*

* Significant stimulation.

Table II. *Effect of heated and unheated bovine serum*

	Litmus milk (control)	Litmus milk +5% serum (heated 1 hr. at 60°)	Litmus milk +5% serum (unheated)
	Streptococcus I		
Str. cremoris BDI	5	5	5
Str. cremoris 18 OJ	2	2	2
Str. lactis 2700	2	4	9*
Str. lactis OJ	2	2	5*
Str. lactis 662	2	2	12*
Str. faecalis 370	6	9	6
Str. faecalis 2707	9	17	12
Str. bovis 2701	15	17	17
Str. diaceti-lactis 4	1	1	1
Str. thermophilus OJ	2	2	2
	Streptococcus II		
Str. agalactiae Z. 62	2	2	3
Str. agalactiae OJ	2	2	2
	Lactobacillus I		
Sbm. casei R 12	3	5	6
L. casei 2–11	12	12	15
Sbm. plantarum 3254	5	5	4
	Lactobacillus II		
Tbm. cereale OJ	>40	>40	>40
L. Delbrücki B	3	3	4
L. acidophilus TC	4	3	3
L. odontolyticus 1406	22	6†	12†
L. bulgaricus BK	18	12	12
L. thermophilus Job B 5	2	2	2
	Lactobacillus III		
Bbm. longum R 1	>40	40	24†
Bbm. breve R 3	>40	24†	12†
L. bifidus 2797	>40	40	>40
L. acidophilus-aerogenes 4304	>40	>40	>40

* Marked inhibition. † Marked stimulation.

Table III. *Effect of* 2 *and* 10% *heated and unheated serum*

	Litmus milk				
		+heated serum		+unheated serum	
	Control	2%	10%	2%	10%
	Streptococcus I				
Str. cremoris OJ	3	2	2	3	2
Str. cremoris OJ	4	14*	8*	11*	8*
Str. cremoris H 739	1	1	1	1	1
Str. lactis OJ	1	1	1	4*	4*
Str. lactis OJ	1	4*	2	5*	4*
Str. lactis 662	4	3	2	12*	12*
Str. lactis 662	4	4	11*	8*	8*
Str. faecalis 370	7	12	12	12	>12*
Str. faecalis 370	8	14	11	11	11
	Streptococcus II				
Str. pyogenes 2400	12	19	19	>36*	>36*
Str. pyogenes Kilner	4	2	1	4	4
Str. agalactiae S. 84	4	3	2	4	4
Str. agalactiae S. 84	6	6	4	11	6
Str. agalactiae OJ	2	2	2	2	2
	Lactobacillus I				
Sbm. casei OJ	12	12	12	12	12
Sbm. casei OJ	6	6	6	6	6
Sbm. plantarum OJ	36	36	12†	36	36
	Lactobacillus II				
Tbm. lactis R 10	2	1	1	2	2
L. acidophilus TC	3	3	3	3	3

* Marked inhibition. † Marked stimulation.

General

On the whole, addition of heated serum, heated blood or unheated blood has little effect on the rate of growth. Litmus and dextrose (0·1%) have no significant effect except that in some instances the small amount of dextrose was found to have a slight inhibitory effect on the lactobacilli. There is thus no evidence for the theory that small concentrations of dextrose may permit the subsequent fermentation of lactose.

Heated serum stimulates to a slight extent the growth of most types, except *Streptococcus* II[1] and *Lactobacillus* III which are markedly stimulated. Unheated blood is without effect, but heated blood stimulates all types, especially *Streptococcus* I and II.

Finally, it may be observed that yeast, and especially yeast and dextrose, are greatly superior to serum and blood even for the pathogenic strains (*Streptococcus* II). The average times of clotting (in days) of the groups are given in Table I.

The behaviour of individual types

Streptococcus I (saprophytes). While *Str. cremoris* is uninfluenced by serum or blood, *Str. lactis* and *Str. glycerinaceus* are inhibited to a slight extent by heated serum and blood. The effect on *Str. faecalis* is rather variable, but *Str. inulinaceus* is markedly stimulated by serum and especially by blood. *Str. bovis*, which is apparently closely related to *Str. inulinaceus*, is less susceptible but is stimulated by heated blood.

Streptococcus II (pathogenic types). It might be expected that these strains, isolated from human and animal infections, would be markedly sensitive to the addition of serum or blood. Strains of *Str. pyogenes* and other pathogenic streptococci of human origin were, in fact, stimulated in varying degrees by serum and blood. Yeast + dextrose was, however, much superior to serum or blood (the nitrogen contents of serum, blood and autolysed yeast were 1·3, 3 and 1·1% approximately).

Str. mastitidis was practically indifferent to serum and blood. Indeed, milk appears to be the most important nutritive for this type. Thus, it has been found, when plating udder samples for mastitis, that the plate containing 1 ml. of added milk may be crowded with colonies of *Str. mastitidis* while lower dilutions (actually 1/30 ml. was used) were devoid of, or contained only one or two, colonies (17).

Streptococcus III (heterofermentative types). This group, which nutritionally is the most fastidious, was unaffected by serum and blood.

Lactobacillus I (mesophilic types). The members of this group were not greatly affected by serum or blood. There was, however, a tendency for *L. casei* to be inhibited by serum and blood, and for *L. plantarum* to be stimulated.

Lactobacillus II (mesothermophilic types). Little effect was found in this

[1] For classification see refs. (13), (14), (15) and (16).

group. *L. odontolyticus* was somewhat stimulated, however, especially by blood, and the single strain of *L. helveticus* was inhibited to a marked extent.

Lactobacillus III (heterofermentative types). Many strains in this group were stimulated by blood.

The effect of heated and unheated serum

Although for media enrichment purposes serum is heated to 60° C. for 1 hr. to destroy bactericidal substances, in raw milk such substances would naturally not be destroyed. Experiments were therefore made with heated and unheated serum. The results are given in Tables II and III.

The most striking results are those for *Str. lactis* and *Str. cremoris*. The latter is unaffected by either heated or unheated serum whereas the former is markedly inhibited by unheated but not by heated serum. This result is perhaps surprising in view of the fact that *Str. cremoris* is generally regarded as more delicate than *Str. lactis*. However, the suggested plant origin of the latter[18] may be a reason for this phenomenon.

In view of the practical importance of this finding, experiments were made under conditions simulating those in practice. *Str. cremoris* and *Str. lactis* were inoculated (1%) into flasks of raw and sterilized milk containing 0, 2 and 5% of unheated and heated serum and blood. The media were incubated at 32° C. and titratable acidities determined at suitable intervals. It was found that under these conditions no significant inhibition or stimulation by serum, heated or unheated, took place. This absence of inhibition may be attributed to the fact that in the earlier tube experiments the inoculum was 0·01% of a milk culture suspended in saline, whereas in the later flask experiments the inoculum was 1% of a milk culture directly added. In the latter case no washing of the cells took place. Since these conditions are similar to those holding for starter propagation and cheese-making it may be concluded that the presence of serum in milk used for starter or cheese-making would not normally result in "slow starter".

Summary

Generally speaking, serum and blood do not have a marked effect on the growth of lactic acid bacteria in milk, although certain types appear to be specifically stimulated or inhibited. Heated serum and, to a greater extent, heated blood, usually stimulate growth.

Unheated serum may inhibit certain types, e.g. *Str. lactis*, when a small inoculum of washed cells is used. Under conditions similar to those in practice, no effect was noticeable. It is unlikely, therefore, that "slow starter" is ever attributable to infiltration of serum or the constituents of serum.

Small amounts of dextrose are without effect on growth (apart from fermentation of the added sugar) and do not initiate the fermentation of lactose.

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222. BACTERIOLOGICAL STUDIES OF SPRAY-DRIED MILK POWDER

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(With 3 Figures)

The great heat to which milk is exposed in roller drying reduces the bacteriological problems to those of the surviving spore-formers or to recontamination with micro-organisms after the powder leaves the rollers. The numbers of bacteria are low and it will be seen from the review by Allen (1) that many of the bacteriological aspects have already been investigated. Roller-dried milk cannot be reconstituted so completely as spray-dried milk. The latter approaches a solubility of 100% and for this reason is increasing in popularity. The extent of heat treatment to which milk is subjected during spray drying is, however, much less than during roller drying, and it might therefore be expected that the bacterial flora of the former would be more numerous and varied. The investigations reported here have therefore been confined to the spray-dried product, since this presented the most interesting and least explored field, few investigations having been conducted with spray-dried milk powder outside the United States.

At the outset it was felt that an insight into the bacteriological quality of spray-dried powder could best be obtained by a general study of samples from the various factories operating in England and Scotland. Seven firms have installed plant for the production of spray-dried milk powder, but during the time that samples were received one plant was not operating and another was drying the constituents of milk which had previously been altered to conform to a "baby food" formula. Finally samples were obtained in 6 oz. sterile bottles from eight factories operated by five firms. Two of these factories were situated in the south-west of Scotland, one in the north of England, and five in the south-west of England, i.e. Somerset, Dorset and Devon. In all, over 400 samples have been examined, and among this number were included samples from plants of the Milkal (2), Kestner (3), and Gray Jensen types. The plants are designated alphabetically throughout. About 10% of the samples were of whole-milk powder and the remainder were made from separated milk. The examination of samples from two plants extended from June 1937 until September 1938, and from the other factories samples were received over the first 8 months of 1938. A drying plant for economic operation is in use from 18 to 22 hr. out of the 24, and samples were received in batches from a day's run of the plant. The first sample was usually obtained in the first hour of drying, another was usually taken at the end of the day's processing, and a third sample was obtained somewhere in the middle of processing. In some

instances four other samples were taken at roughly regular time intervals between the first and last samples. It will be seen, therefore, that any conclusions regarding the bacteriological quality of the powders are based on fully representative samples since they are derived from different types of plant, situated in different localities and sampled throughout cold and warm seasons and at different stages in the day's run.

A. EXPERIMENTAL METHODS

I. *Method of reconstitution of spray-dried powders*

The reconstitution of samples for analysis presented two difficulties, viz.

(1) the avoidance of contamination, and

(2) the preparation of a representative sample.

Regarding (1) there are two points of contamination, (*a*) at weighing, and (*b*) on reconstituting.

Quick weighing of a large sample on a rough balance minimized contamination at weighing. The prevention of contamination on reconstitution was more difficult. Spray-dried powder, although almost completely soluble when mixed with water, behaves like cornflour, and unless it is first stirred thoroughly into a paste, lumps of undissolved powder make it impossible to get a uniform solution. A sterile glass rod was used to rub the powder into a paste in a wide test-tube, but it was not successful and the technique could not be considered satisfactory. The same criticism also held when a pestle and mortar were used to make the paste. Hence a closed bottle containing water and some beads was eventually adopted.

As regards (2), the necessity for having a representative sample was attained by (*a*) using a large sample, and (*b*) thoroughly reconstituting.

It will be seen that the technique devised to avoid contamination incidentally also met these two points. The American Dry Milk Institute (4) recommend weighing the powder directly into a sterile bottle containing the water blank and furnished with a sterile stopper. Other workers have weighed the powder directly into a sterile bottle and Ringer's solution has been added afterwards. These methods of weighing have obvious disadvantages, and in addition it requires considerable shaking to get a completely dissolved powder, although the addition of lead shot has recently been suggested (4) to facilitate solution. It should be noted also that dilution blanks containing $N/60$ sodium or lithium hydroxide have been suggested (5) for the analysis of milk powder when difficulties are experienced in counting plates after incubation owing to undissolved flakes or particles of powder. The presence of undissolved flakes is common in plates made from roller-dried powder, but no difficulties were encountered during the examination of the more soluble spray-dried skimmed milk powders, and hence weak alkaline dilution blanks were not used in this study. However, with spray-dried whole-milk powder, fat particles rendered plates of the 1/10 and 1/100 dilutions difficult to estimate with accuracy.

The method of reconstitution finally adopted was to weigh out 20 g. of powder into a pair of sterile Petri dishes, and transfer the powder through a sterile paper cone into a glass bottle containing 180 ml. of sterilized water. The capacity of the bottle, which was secured by a screw cap, was about 300 ml., and it contained about a dozen glass beads ($\frac{1}{8}$ in. diameter). By vigorous shaking all the powder was dissolved in from 1 to 3 min. Twenty g. of powder added to 180 ml. of water increase the total volume of reconstituted milk to between 190 and 195 ml. Hence it will be seen that the reconstituted milk was roughly a 10% solution, and for all practical purposes 1 ml. could be taken as equal to 0·1 g. and could be considered as the 1/10 dilution. Incidentally this reconstituted milk contains about 9·5% solids-not-fat which corresponds to the composition of separated milk itself.

II. *General methods of examination of samples*

The following tests were carried out on all the powders:

(i) Plate count was determined by the method described in *Bulletin* 46 of the Ministry of Agriculture(6) on milk agar at 37 and 55° C. Nearly all the dilutions were prepared in duplicate and were not made any lower than 1/1,000,000. It was necessary to incubate all plates at both temperatures for at least 3 days before the count was taken; even then many of the colonies were very small. In an attempt to improve the technique plates were prepared from twenty-four samples in this way, and were compared with a series to which $\frac{1}{2}$ ml. of defibrinated horse blood had been added as the agar was poured. It was found that the colonies on the plates to which blood had been added were usually larger and easier to count and that the number was somewhat higher. The disadvantages of this method for routine purposes seemed to outweigh the advantages, however, especially as, when combined with the milk agar, the blood was haemolysed, owing to the low *p*H, and any haemolysing organisms present could not be estimated.

(ii) The methylene-blue reduction test was applied at 37 and 55° C. as described by Wilson(7) for whole milk.

(iii) The tubes of milk used for the reduction test were examined after 24 hr. incubation, and the type of fermentation noted.

(iv) 1 ml. of the reconstituted milk, and in some instances 10 and 20 ml., were tested by the presumptive coliform test(6) at 37° C.

(v) 1 ml. of the reconstituted milk was also added to litmus milk and incubated at 37° C. for 3 days, as it was considered that changes in the litmus milk would provide information as to the type of flora present.

(vi) Keeping quality tests(6) were carried out at 60° F. The reconstituted milks could not be graded from the first perceptible taint up to clotting, as any slightly developed taint was masked by the somewhat cooked and often acid flavour of the reconstituted milk itself. The cooked and/or acid flavours were more marked in the milk reconstituted from skimmed-milk powder than in that made from whole-milk powder. The keeping quality was therefore judged from the time of reconstitution until clotting took place on boiling or until a clot was formed in the bottle.

The following tests were carried out on some samples only:

(vii) The testing was repeated on certain samples after the reconstituted milk had been stored at 60° F. for 24 hr.

(viii) About forty powder samples were retained at laboratory temperatures and about 100 retests were made after they were $1\frac{1}{2}$, 3, 6, 8 and 12 months old.

(ix) A further series was made up in cans, each of which held about 200 g. of separated milk powder, from a large bulk sample and was stored at 40° C., 22° C., cold-room temperature (about 10° C.) and in a cold store (about 4° C.). Half the sample cans were evacuated of air and refilled with an inert gas, nitrogen. In order to do this, special equipment was supplied by the Metal Box Company, Worcester. The cans were examined at stated intervals by the methods already outlined.

(x) 1 ml. quantities of a few of the reconstituted samples were plated on beer wort agar, which had been buffered to a *p*H of 3·5 with citric acid and potassium citrate. This medium was selective for yeasts and moulds. The plates were incubated at 22° C.

III. *Method of isolation of organisms*

About 1700 colonies have been picked off the agar plates used for the plate count test. These colonies were inoculated into litmus milk, since growth in this medium would be indicative of changes which might be expected in milk products made from the powder. The tubes were incubated at 37 or 55° C. according to the temperature at which the plate had previously been incubated. The changes in the litmus-milk cultures were recorded for about a week, and most of the cultures, where no change was noted, were examined microscopically to ascertain whether growth had occurred. All the colonies from a plate or a portion of a plate were picked off, so that the cultures obtained were representative, and about 180 of them were purified and examined in detail.

B. RESULTS AND DISCUSSION

I. *Bacteriological quality of spray-dried milk powder*

(1) and (2) *Plate count and methylene-blue reduction test.*

It will be seen from Table I that the arithmetic "weighted" mean of the plate count at 37° C. was 4,363,000 per g. of powder, and if this is considered as reconstituted milk it will approximate to a plate count of 400,000 per ml., which does not compare very favourably with good quality raw or pasteurized milk. Wide variation in the plate count was shown, and individual results varied from 1400 to 149,000,000 per g. By using the logarithm of the plate count at 37° C. the extreme values are levelled out to a certain extent and the mean value was then found to be equivalent to a count of 800,000 per g. The "weighted" mean methylene-blue reduction time at 37° C. was 8·1 hr., and varied from 3 to 14 hr. or more.

The plate counts at 55° C. also varied widely, and ranged from less than 100 to 1,300,000 per g. The methylene-blue reduction times were much higher than at 37° C. The lowest time was 4½ hr., but over 30% of the samples required 12 hr. or more before decolorization occurred.

The factors responsible for these variations will be dealt with later.

(3) *Fermentation test.*

No definite or uniform relationship was established between the fermentation reactions of the methylene-blue tubes examined after 24 hr. incubation and the plate count and reduction tests. Useful data were, however, occasionally obtained regarding the number and activity of thermophiles present when these tests were conducted at 55° C. Typical samples given in Table II illustrate this point. G 1–3 and C 19 and 20 show how deterioration in the results of the fermentation test may forecast an increase in the number of thermophiles or a change in the activity of the organisms (E 89–91). It was apparent from the results that some factories had more thermophiles present than others, and that the activity of the organisms also varied. Factory C, for instance, usually

Table I

Factory	Plate count at 37° C. per g.				Log of plate count at 37° C.					
			Range						Range	
	No. of samples	Mean	Min.	Max.	No. of samples	Mean	Standard deviation	Coeff. of variation (%)	Min.	Max.
A	24	4,265,000	46,000	20,200,000	24	6·05	0·7085	11·70	4·66	7·31
B	63	1,535,000	6,000	24,800,000	63	5·74	0·7280	12·69	3·78	7·39
C	66	19,800	1,500	129,000	66	4·07	0·3911	9·60	3·18	5·11
D	90	12,250,000	28,000	149,000,000	90	6·19	1·321	21·34	4·45	8·17
E	138	3,388,000	1,400	45,500,000	138	5·93	0·9132	15·40	3·15	7·66
F	12	305,500	22,000	1,170,000	12	4·89	1·946	39·70	4·34	6·07
G	12	106,300	15,600	450,000	12	4·77	0·6124	12·84	4·19	5·65
Total	405	4,363,000*	1,400	149,000,000	405	5·65*	0·8161	14·45	3·15	8·17

Factory	Methylene-blue reduction test at 37° C. in hours					
					Range	
	No. of samples	Mean	Standard deviation	Coeff. of variation (%)	Min.	Max.
A	23	8·18	2·033	24·9	4·5	12·5
B	63	7·65	1·217	15·9	5·0	11·0
C	61	11·03	1·482	13·45	7·5	14·0
D	94	6·84	1·426	20·8	3·0	10·0
E	139	7·81	1·767	22·8	3·5	12·5
F	11	9·45	1·094	11·6	7·5	11·0
G	12	8·54	1·244	14·6	6·0	11·0
Total	403	8·13*	1·805	22·3	3·0	14·0

* Weighted mean.

Table II

Sample	No.	Plate count at 55° C.	M.B.R.T.* at 55° C.	Fermentation reactions after 24 hr. at 55° C.				
				Unchanged	Coagulated	Whey	Digestion	Gas
G	1	100	>14½	+	.	.	.	.
	2	2,400	13½		+	.	.	.
	3	63,600	8½	.	+	.	.	.
C	19	26,000	>18½	+	.	.	.	.
	20	55,000	11½	.	+	.	.	.
E	89	1,030	9½	.	+	+	.	.
	90	1,430	4½	.	+	+	+	+
	91	560	6	.	+	+	.	.

* Methylene-blue reduction test.

had from 1000 to 10,000 per g., although the consistency of the milk in the fermentation test was nearly always unchanged. It is probable, therefore, that the fermentation test is a better indication than the actual plate count at 55° C. of any difficulties which might be expected in the plant operation owing to increased acidity which had been produced by thermophiles.

(4) *Presumptive coliform test.*

When 1 ml. quantities of the reconstituted milk were tested by the presumptive coliform test about 10% gave positive results. A few samples which gave negative results with 1 ml. showed positive reactions when tested in addition in 10 and 20 ml. quantities. It would seem, however, that the latter is too stringent a test, since 1 ml. quantities are considered sufficient to judge the quality of ordinary raw and pasteurized milk. It should be noted that positive tests were often due to the growth of anaerobic spore-formers and not to organisms of the coliform group. Crossley (8), working with the powder from two similar plants, reported that 65% of the positive presumptive coliform tests are due to anaerobes, and that the positive presumptive coliform tests actually caused by coliform organisms were more frequent in freshly made than in stored samples. From additional tests it did not, however, appear that the percentage of positive results due to anaerobes would normally be so high as Crossley reports, provided that the survey is (as in the present investigation) spread over samples from a large variety of plants.

(5) *Yeast and mould count.*

Since the heat of processing is sufficiently severe to kill yeasts and mould spores, it is generally held that the sieving and packing of the powder constitute the only serious source of contamination with these organisms. However, the number of yeasts and moulds in the samples which were examined by plating on beer-wort agar was found to be practically negligible.

One special sample was encountered which had a mouldy odour and taste, and a mould of the *Penicillium* genus was found to have grown near the surface, where the moisture content of the powder was very high. It is probable that this was due to damp gaining entrance to the barrel.

II. *Keeping quality of reconstituted milk and effect of storage at* 60° F.

It has sometimes been held that reconstituted milks only keep in a palatable condition for a very short time as compared with fresh milk. It was felt therefore desirable to investigate this point. It has already been stated that the keeping quality could not be determined by taste, and the figures in Table III refer to the time between reconstitution and the formation of a clot on boiling or in the bottle. In order to convert these values to the actual keeping quality, i.e. to the time of development of an undesirable flavour, half a day at least should be deducted. If this deduction is made, over 90% of the samples remained sweet for between $2\frac{1}{2}$ and $3\frac{1}{2}$ days, which allows ample time for the use of reconstituted milk for liquid consumption. Thus it appears that the belief that reconstituted milks have a short life is unwarranted.

Table III. *Showing percentage of samples which clotted on boiling or were coagulated in the bottle after different periods of storage at* 60° F.

	Days of storage							
Factory	1	$1\frac{1}{2}$	2	$2\frac{1}{2}$	3	$3\frac{1}{2}$	4	$4\frac{1}{2}$
A	—	—	—	10	43	33	14	—
B	—	—	—	—	37	38	25	—
C	—	—	—	—	18	32	44	6
D	1	1	1	8	53	24	12	—
E	—	—	1	5	54	22	17	1
F	—	—	—	—	25	50	25	—
G	—	—	—	25	50	16	9	—
Total	0·25	0·25	1	4·5	44	28	21	1

Examination of the results obtained with the samples which were retested after 24 hr. storage at 60° F. showed that the plate count at 37° C. had generally increased and the reduction time had decreased, but that the changes were very much less than would have been expected. The reduction test did, however, appear to be the more sensitive indication of the additional ageing of the milk, as will be seen from Table IV. The samples tabulated were chosen at random. It will be seen that the reduction time was approximately halved after 24 hr., whereas the increase in the plate counts was less regular. It is probable that other factors, such as the operation of a long initial lag phase before increase of numbers occurs, as well as the tendency for the organisms to clump when multiplication commences, may have affected the counts but not the activity of the organisms as shown by the reduction test. At 55° C. there was less uniformity in the results. In the main the tests showed no changes, a result which would be expected at a storage temperature too low for thermophilic growth to occur.

It is apparent that the flora of reconstituted milk does not increase as rapidly as that of raw milk at 60° F., and this must be given consideration if the quality of the reconstituted milk which has been made some hours is to be assessed by the plate count alone. The work of Ayers *et al.*(9) showed that,

Table IV. *Showing tests on milk powder when first reconstituted and after 24 hr. storage at 60° F.*

Sample no.	Time after reconstitution when tested hr.	Plate count at 37° C.	Reduction test at 37° C.	Plate count at 55° C.	Reduction test at 55° C.	Presumptive coliform test
131 A	0	1,070,000	7	20,800	>8	Acid
	24	730,000	5	8,600	7	Acid + gas
109 D	0	20,200,000	4	—	9½	Acid
	24	25,000,000	1½	—	7½	Acid
114 D	0	160,000	6½	340	9½	Acid
	24	690,000	3½	220	9½	Acid
73 D	0	1,690,000	6½	40,000	5½	Acid
	24	9,200,000	4½	60,000	6	Acid + gas
48 D	0	1,310,000	6½	—	6	Acid
	23	550,000	7	—	5	Acid
34 D	0	5,800,000	8½	30,000	8	Acid
	28	8,700,000	7½	18,800	8	Acid
142 E	0	94,000	12	—	14	Acid
	24	270,000	7	—	10½	Acid
140 E	0	5,200,000	6½	70,000	6	Acid
	28	12,600,000	3	45,000	7	Acid
125 E	0	4,600,000	7	34,000	10	Acid
	24	3,280,000	4	20,500	>8½	Acid + gas
89 E	0	100,000	9½	1,030	8½	Acid
	24	224,000	7	90	>7½<10½	Acid
79 E	0	246,000	9	90	10	Acid
	24	288,000	5	210	9½	Acid

averaging the results of sixteen clean milk samples, there was a 38-fold increase in count after 24 hr., i.e. from 3243 to 123,562 per ml., and the average result of thirteen samples of dirty milk increased 63-fold, i.e. from 707,761 to 48,550,923 per ml. In comparison, a 5-fold increase in the plate count of the reconstituted milk was exceptional, while in some samples there was actually a reduction in the count 24 hr. after reconstitution.

III. *Bacterial flora of dried-milk powder*

(1) *Litmus-milk reactions of micro-organisms at* 37° C.

Examination of the litmus-milk media which had been inoculated with 1 ml. of reconstituted milk after 3 days' incubation at 37° C. showed that in all cases the predominating flora at this temperature rendered the milk acid and coagulated it. The acidity produced was sufficiently high to cause the milk to "whey off" in 60% of the samples. Roughly 40% of these cultures also showed gas formation and about 20% appeared to be partially digested. It was, however, often difficult to differentiate whey production and digestion, particularly if the curd had shrunk and was broken up by gas.

(2) *Classification of cultures obtained from plates incubated at* 37° C.

Tables V and VI show that the majority of organisms which are present in spray-dried milk powder produce souring readily. 80·5% of the 1304

Table V. *Litmus milk reactions of cultures obtained from spray-dried milk powder plates incubated at* 37° C.

		Litmus milk										
		Unchanged								Peptonization		
			Microscopic examination showed			Acid coagulation						
Factory	Total no. of colonies inoculated into litmus milk	No further examination made	No bacteria present	Bacteria present	Acid	Total	Litmus reduction also	Whey production also	Alkali	Alkali	Unchanged in reaction	Slightly acid reaction
A	208	—	16	5	15	170	43	84	—	—	—	2
B	181	4	3	3	7	159	108	47	—	—	4	—
C	184	33	18	5	7	113	64	21	—	4	3	1
D	303	12	13	3	7	269	100	75	—	1	—	—
E	323	4	30	2	7	274	72	81	—	6	—	—
G	105	12	9	15	4	64	16	39	—	—	—	1
Total	1304	65	89	33	47	1049	403	347	—	11	7	4
%	—	5·0	6·9	2·3	3·6	80·5	31·0	27·0	—	0·9	0·5	0·3

Table VI. *Litmus milk reactions of cultures obtained from spray-dried milk powder plates incubated at* 55° C.

		Litmus milk							
		Unchanged							
			Microscopic examination showed			Acid coagulation			
Factory	Total no. of colonies inoculated into litmus milk	No further examination made	No bacteria present	Bacteria present	Acid	Total	Litmus reduction also	Whey production also	Peptonization slightly acid reaction
A	29	17	8	—	—	3	—	—	1
B	134	16	8	3	10	97	46	14	—
C	147	10	—	—	22	115	84	2	—
D	42	—	3	—	—	39	—	—	—
E	7	—	—	—	—	7	7	—	—
G	19	—	—	—	—	19	—	—	—
Total	378	43	19	3	32	280	137	16	1
%	—	11·4	5·0	0·8	8·5	74·0	36·2	4·2	0·3

cultures picked off the plates incubated at 37° C. and 74% from the plates incubated at 55° C. produced acidity and coagulation in litmus-milk media.

Macey (10) also showed a preponderance of acid-forming bacteria from milk powder, but his figures are lower unless the percentage of acid peptonizing cultures are included with those producing acid coagulation. This is, however, scarcely justifiable, viz.

Percentage of cultures producing	weak acid	26·0%
,,	acid coagulation	26·8%
,,	acid peptonization	12·8%

Since the production of acid and coagulation in milk media was the most characteristic property of the organisms isolated, most of the cultures examined in detail were from the litmus-milk inoculations which showed these changes.

The 124 cultures obtained in this way, originating from colonies which grew on agar plates incubated at 37° C. and produced acid coagulation in milk, were, with three exceptions, all streptococci. These have been classified according to the suggestions given by Sherman (11) in his review on the streptococci and, apart from one strain in the "lactic" division, all fall into the "*viridans*" and "*enterococcus*" divisions.

Primary grouping. In this grouping two of the more important "tolerance tests", namely, the growth in media of *p*H 9·6 and growth in the presence of 6·5% sodium chloride, have not given as sharp a line of distinction as the data given in Sherman's review suggests.

In glucose-agar stabs of *p*H 6·8 the growth of the streptococci examined was invariably heavy, and throughout the line of inoculation after a period of from 1 to 3 days' incubation at 37° C., but when the *p*H of the same media was raised to 9·6 growth was in several instances less definite. Occasionally slight growth occurred at the top end of the stab after 2–3 days, but for some cultures this was variable. When the streptococci were inoculated into 2% glucose broth containing 6·5% salt a few cultures placed later in the "*enterococcus*" division only grew very lightly. It was possible to pick out the cultures which developed as heavily as they would have done in the same media without salt, and also to select the large number of cultures which failed to develop at all. In between these extremes of development, however, the growth of about 15% of the "*enterococcus*" strains was less marked. Tolerance of 6·5% salt was determined by changes in the *p*H of the glucose-broth media, since all the strains examined fermented glucose. In addition growth was indicated by an increase in the turbidity of the broth.

Detailed analyses of the strains of streptococci isolated are given in Tables VII and VIII. It will be seen from Table VII that 66% of the cultures were classified into the "*viridans*" division and 33% into the "*enterococcus*" division. All these organisms grew at 45° C., many cultures producing sufficient acidity to coagulate milk in 12 hr. at this temperature, and all survived 60° C. for 30 min. Crossley (3), working with the powder from one type of plant only,

has also reported that acid-forming bacteria nearly always predominate in the spray-dried milk powder and that thermoduric cocci form the bulk of the flora.

Table VII. *Classification of cultures causing acid coagulation of litmus milk obtained from agar plates of milk powder incubated at* 37° C.

Division	Group of species	No. of cultures isolated			Percentage	
Pyogenic	*Str. pyogenes* *Str. mastitidis* *Str. equi* "Animal pyogenes" The "Human C" "Minute haemolytic" Group G streptococci Group E streptococci Group H streptococci	None			—	
Viridans	*Str. salivarius*	80	1		0·8	66·1
	Str. equinus		None		0	
	Str. bovis		6	3*	5·0	
	Varieties of *Str. bovis*		4	3*	3·3	
	Str. thermophilus		69		57·0	
Lactic	*Str. lactis*	1			0·8	0·8
	Str. cremoris					
Enterococcus	*Str. fecalis*	40	8	4*	6·6	33·1
	Str. liquefaciens		None		0	
	Str. zymogenes		None		0	
	Str. durans		32	2*	26·5	
Total no. of cultures examined		121			100	

* Cultures which showed sufficient variation from fixed type characteristics to make their inclusion in the strain subject to question; such cultures have, however, been included in the percentage figures.

The differential tests on which the grouping was based is given in Table VIII under each species. A number of strains, referred to later in detail, gave occasional reactions to some of the tests which were dissimilar to those which would be expected for a particular group as a whole, although it appeared from the other tests that they should be placed in that group. The exceptional reactions of these cultures have been given in the reaction column as an extension.

Although seven out of eleven possible strains of the non-pyogenic streptococci were recovered from spray-dried milk powder, 83·5% of the cultures were restricted to strains of *Str. thermophilus* or *Str. durans*, more than twice as many strains of the former being isolated as of the latter.

"*Viridans*" *division.* Regarding the differentiation of the streptococci placed in this division, the greatest number, i.e. 86%, of strains were of *Str. thermophilus* and no difficulty was encountered in classifying them by the method advocated by Sherman (11), except in respect of their tolerance to 2% sodium chloride. 2% glucose broth, in which heavy growth occurred with these streptococci, was again used as the basic media for the 2% salt tolerance tests, and it was noted that about 35% of the strains of *Str. thermophilus* grew slightly and irregularly in this salt broth. Sherman (12) reports that as the total

Table VIII

	Viridans division								*Enterococcus* division								
					Varieties of												
	Str. salivarius	*Str. equinus*	*Str. bovis*		*Str. bovis*		*Str. thermophilus*		*Str. fecalis*		*Str. liquefaciens*	*Str. zymogenes*	*Str. durans*				
No. of strains ...	1	0	3	3*	1	4*	45	24	4	4	0	0		24*	6*	1*	1*
Primary characteristics																	
Haemolysis	−	−	−	.	−	.	−	.	−	.	−	+	+	24 −	6 −	1 −	1 −
Growth at 10° C.	−	−	−	.	−	.	−	.	+	.	+	+	+	.	.	.	.
45° C.	±	+	+	.	+	.	+	.	+	.	+	+	+	.	.	.	.
Growth in the presence of:																	
6·5% NaCl	−	−	−	.	−	.	−	.	+	.	+	+	+	.	.	.	.
*p*H 9·6	−	−	−	.	−	.	−	.	+	.	+	+	+	.	.	.	.
0·1% methylene-blue	−	−	−	.	−	.	−	.	+	.	+	+	+	.	.	.	.
Survival at 60° C. for 30 min.	−	±	+	.	+	.	+	.	+	.	+	+	+	.	.	.	.
Strong reduction	−	−	−	.	−	.	−	.	+	.	+	+	−	.	.	.	.
Additional characteristics																	
Growth in 2% NaCl	+	+	+	.	+	.	−	24 ? +	.	.	.	.	.	.	.	.	.
Starch hydrolysed	−	±	+	.	−	.	−	.	−	.	−	−	−	.	.	.	.
Aesculin split	±	+	+	3 −	+	2 + 2 −	−	.	+	.	+	+	+	.	.	.	.
Gelatine liquefied	−	−	−	.	−	.	−	.	−	.	+	±	−	.	.	.	.
Milk curdled	±	−	±	.	±	.	+	.	+	.	+	+	+	.	.	.	.
Final *p*H in glucose broth	5·4 4·0	4·5 4·0	4·5 4·0	. .	4·5 4·0	. .	4·5 4·0	. .	4·5 4·0	. .	4·5 4·0	4·5 4·0	4·5 4·0	. .	. .	. .	. .
Acid from: Maltose	+	+	+	.	+	.	−	.	+	.	+	+	+	.	.	.	.
Sucrose	+	±	+	.	+	2 + 2 −	+	.	±	3 + 1 −	+	+	−	21 − 3 +	1 − 5 +	1 +	1 −
Lactose	+	−	+	.	+	.	+	.	+	.	+	+	+	.	.	.	.
Raffinose	±	±	+	.	+	3 + 1 −	±	.	±	2 + 2 −	±	±	−	.	.	.	.
Glycerol	−	−	−	.	−	.	−	.	±	3 + 1 −	+	+	−	.	.	1 +	1 +
Sorbitol	−	−	±	.	−	1 + 3 −	−	.	+	3 + 1 −	+	+	−	.	6 +	.	1 +

* Strains which showed variations from type species of a particular group but which have been included in that group for classification purposes.

number of cultures examined by the 2% NaCl tolerance tests has been small, he would be surprised to find on the examination of greater numbers that all strains of *Str. thermophilus* were inhibited by this amount of salt. He suggests that as the salt tolerance of *Str. thermophilus* is a unique property a slight increase in the percentage of salt might be better as an empirical test for this group.

"*Enterococcus*" *division.* None of the cultures classified as *Str. durans* was able to haemolyse defibrinated horse blood on poured (not streaked) agar-plate culture, although blood haemolysis is considered by Sherman (11) to be one of the primary characteristics of *Str. durans*. Sherman (12) reports more recently that a few strains of *Str. durans* have been encountered in his laboratory which are non-haemolytic, and he considers that it is logical to anticipate the existence of such strains, since the same type of relationship exists between the other so-called species in the "*enterococcus*" division. It should be noted that nine of the strains classified as *Str. durans* produced acid from sucrose. Sherman in his review characterizes *Str. durans* cultures as failing to ferment sucrose, but more recently Smith & Sherman (13) report that about 25% of the strains of *Str. durans* isolated from human faeces produce acid. Six strains also classified as *Str. durans* fermented sorbitol, which is usually considered to be unattacked.

(3) *Classification of milk-curdling cultures obtained from plates incubated at* 55° C.

Thirty cultures producing an acid coagulum in litmus milk have been obtained from colonies picked off agar plates previously incubated at 55° C. and examined in detail. All of these cultures were found to be obligate thermophilic sporeformers which grew strongly at a temperature of from 55 to 60° C. but not at 37° C. Acid production was very fast and it was not uncommon for litmus milk to be coagulated in 18 hr. at 55° C. This type of thermophilic sporeformer was first encountered in February 1936 when Factory D, which works on a continuous system, encountered difficulties from (1) milk clotting in the nozzles of the spray and in the high-pressure pipe line supplying it at temperatures of 130–160° F., (2) coagulation of batches of milk in the holding tanks at 160° F., and (3) high acidity in some of the dried powder. From tests of the incoming milk and of the milk throughout processing it was seen that, although the thermophilic organisms were present in small numbers in the incoming milk as might be expected in any similar bulk of mixed separated milk, they increased throughout the operation of the plant, reaching very high numbers in the final concentrated milk which is pumped to the spray. It was apparent that thermophiles were the cause of the trouble, for all the cultures isolated grew at 70° C. and produced sufficient acidity to coagulate milk in under 15 hr. The plant was running for about 20 hr. out of 24, and some of the incoming milk was held at 160° F. for the whole of that time. Hence the conditions in the plant were ideal for the proliferation of the organisms, and consequently it is not surprising that difficulties from the acid produced by

them arose. The plant was not sterilized as thoroughly as the conditions of processing necessitated, and this, together with the fact that some of the concentrated milk remaining from the previous day was dried first on the following day's operation, obviously seeded the plant from day to day.

A study of the characteristics of the thirty cultures mentioned above showed that they were all strains of *B. calidolactis* (14). This organism was observed by Hussong & Hammer (14) to cause the coagulation of separated milk which was held at 71–77° C. before drying, and it has also been reported by them to coagulate evaporated milk on storage at high temperatures.

Prickett (15) found from an extensive examination of different samples, which included raw and pasteurized milk, milk powder, milk-stone and some cattle foods, that *B. calidolactis* was one of only two thermophilic sporeforming bacteria which he isolated that produced an acid curd in milk. *B. thermoliquefaciens*, the other sporing thermophile reported by him to curdle milk, was very similar to *B. calidolactis*, except that it liquefied gelatine and always reduced nitrate, whereas reduction of nitrate by *B. calidolactis* is variable.

The cultures examined agreed with the description of *B. calidolactis* already recorded (14) except that some strains grew more readily on agar slants. This has also been noted by Prickett (15). In addition it was found that after several cultivations in the laboratory on agar slants the extent of growth of some strains which had grown scantily at first increased, and that the growth on milk-agar slants was usually good.

(4) *Other organisms examined.*

The numbers of bacteria causing peptonization at 37° C. were very low and almost negligible at 55° C. The ten cultures causing peptonization at 37° C., which were examined in detail, were all aerobic sporeformers of the *B. subtilis* type which are common in most dairy products. The strains isolated were as follows:[1]

B. subtilis	4
B. licheniformis	4
B. pumilus	2

In addition one aerobic sporeforming thermophile was isolated, which produced large terminal spores readily on agar but only digested milk very slightly after several days. The identity of this organism was not definitely established, and since it did not appear to be very common it is probably not of commercial importance.

Examination of the plates throughout the analysis showed that aerobic sporeformers were not present in large numbers and seldom occurred in plates made from less than 0·001 g. of powder at 37° C. or 0·1 g. at 55° C. Thus they were found to be more prevalent in the lowest count samples and were particularly evident in the samples from Factories C and B.

[1] The basis of classification of the aerobic sporeformers is discussed in the succeeding paper (20).

About 14% of the inoculations from agar plates held at 37° C. which were made into litmus milk did not change the media visibly; 9% were further examined microscopically for the presence of bacteria. About 7% of these inoculations showed no growth, so that either the inoculations had been faulty or the organisms did not grow in milk. The organisms which grew in the milk at 37 and 55° C., but caused no change under the conditions of test, were a mixture of cocci and bacteria, and since they have probably little significance as far as the reconstituted milk is concerned they were not examined further.

IV. *Factors affecting variability in bacteriological quality of spray-dried milk powders*

As already indicated, the bacteriological quality of the spray-dried powders was found to vary widely, whether assessed by the plate count or by the methylene-blue reduction test. It was felt desirable to study the figures in more detail in order, if possible, to determine the chief factors responsible for the observed variations.

(1) *Variations in the powder of different factories.*

Table I shows that there were wide variations in the mean plate counts from one factory to another, extending from roughly 20,000 per g. with Factory C to over 12,000,000 per g. with Factory D. The mean values of the reduction times showed corresponding variations, the figures varying from 11·03 hr. with Factory C to about half this period (6·84 hr.) with Factory D. It is obvious therefore that differences in the quality of the incoming milk supply and/or the technique of manufacture at the various factories caused considerable variations in the quality of the final product.

(2) *Variations in the powder from the same factory.*

Apart from variations in the mean counts from one factory to another, marked differences were found in the ranges of the counts obtained at each individual factory. These are to some extent apparent in the standard deviations and coefficients of variation of the logarithms of the plate counts (Table I). But they are perhaps most clearly shown from a study of the maximum and minimum figures, which give the extreme ranges of the counts. Thus, whereas the counts for Factory C varied only from 1500 to 129,000 per g., and for Factory G only from 15,600 to 450,000 per g., Factory A showed a range of from 46,000 to 20,200,000 per g. and Factory D the extreme values of 28,000 to 149,000,000. Corresponding variations were found in the reduction times. Apart, therefore, from any general differences in the technique of manufacture between the different factories, it may be said that in some factories there must have been a complete lack of consistency in the day-to-day management of the plant.

(3) *Variation in the powder from the same factory during the same day's operation.*

It might be expected that as many of the factories were operated continuously during the day's run, the bacteriological quality would deteriorate through the multiplication of thermophilic or thermoduric bacteria during processing. Such deterioration has, of course, been frequently observed in large-scale pasteurizing plants when tanks have been used for long periods without intermittent sterilization. Thus powder produced during the first few hours of plant operation might be expected to be of a higher quality than that produced at the end of the day's run. The mean values of the plate counts and reduction times at 37 and 55° C. (Table IX) of powders sampled at the beginning of processing and towards the end of the same day's manufacture demonstrate that there is, in fact, some indication of a falling off in bacteriological quality, though the results are by no means consistent. It will be seen,

Table IX. *Comparison of results of tests on powder sampled at the beginning and at the end of the same day's operation*

		Log plate count at 37° C.		M.B.R.T. at 37° C.		Log plate count at 55° C.		M.B.R.T. at 55° C.	
Factory	Samples	No. of samples	Mean	No. of samples	Mean	No. of samples	Mean	No. of samples	Mean
A	First	7	5·80	6	9·17	7	4·13	5	6·90
	Last	7	6·02	6	7·83	7	4·29	5	8·70
B	First	19	5·81	19	7·55	18	3·55	19	10·71
	Last	19	5·74	19	7·68	18	3·66	19	9·47
D	First	17	6·21	18	6·92	16	3·85	13	9·15
	Last	17	6·14	18	6·85	16	4·40	13	7·27
E	First	28	5·53	29	7·80	17	3·58	22	9·36
	Last	28	6·12	29	7·53	17	3·75	22	8·68
F	First	4	5·52	3	9·50	2	2·67	1	12·5
	Last	4	5·27	3	8·50	2	2·50	1	12·5
G	First	4	4·54	4	9·38	3	2·07	—	—
	Last	4	5·13	4	7·63	3	3·63	—	—
Total	First	79	5·71	79	7·78	63	3·66	60	9·46
	Last	79	5·93	79	7·53	63	3·90	60	7·58

however, that the results of the plate counts made at 55° C. are definitely more consistent than those made at 37° C., which appears to indicate (as would be expected) that any increase in count is attributable to thermophilic and thermoduric types.

It is doubtful, however, whether the figures in Table IX can be adequately interpreted without reference to the exact method of processing involved at each individual factory. This point may be illustrated by reference to Factory E. At this factory the day's operations usually began by drying some of the precondensed milk which had remained from the previous day's manufacture. This practice increased the economy of working, but it is clear that the old precondensed milk (especially when inadequately cooled between the two different days' operations) would be heavily contaminated with both ordinary milk flora and thermoduric bacteria. Hence the first powder to be dried would probably have an abnormally high count; this count would then be diluted as

fresh milk entered the precondenser, but the count of the latter (being well seeded with thermoduric bacteria from the old precondensed milk) would again gradually increase as the day's run progressed. That such a sequence actually occurred in Factory E was shown in tests carried out on samples of powder taken at intervals throughout a single day's run, the results of the tests being as follows:

Factory E sample no. July 1938	Hours after start of manufacture when sampled	Plate count at 37° C.	Reduction test at 37° C.	Plate count at 55° C.	Reduction test at 55° C.
103	0	36,000,000	4½	1300	7½
104	2	8,900,000	7½	370	10½
105	4	5,800,000	7½	550	10½
106	6	1,310,000	7	1700	8½
107	9	2,150,000	6½	2600	7
108	11	5,300,000	5½	3200	7½

Such a regular sequence is probably exceptional, since many adventitious factors may affect the bacteriological quality of the product at any given time. The above results serve, however, to show that while the figures given in Table IX cannot be taken at their face value, they probably tend to underestimate rather than to over-estimate the detrimental effect of continuous operation on the quality of the powder.

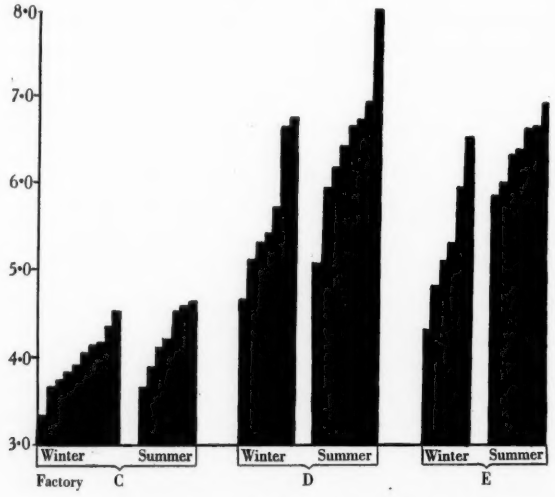

Fig. 1. Logarithm of the plate count at 37° C.

(4) *Variation according to season of year.*

An examination of the results obtained on samples of powder obtained from individual factories shows that seasonal variations in quality only occur where the general bacterial standard is normally poor. This fact is illustrated, in the forms of histograms, in Figs. 1–3, where the average figures for a series

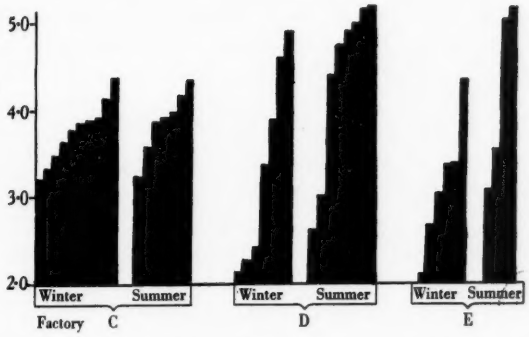

Fig. 2. Logarithm of the plate count at 55° C.

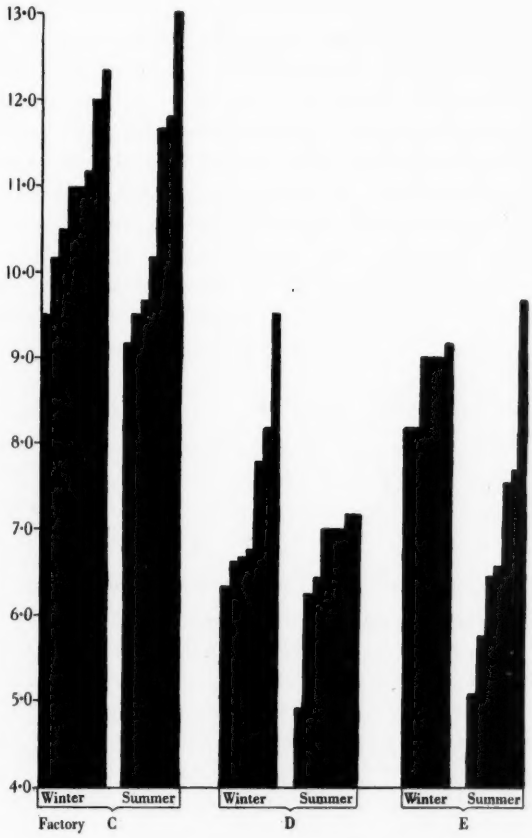

Fig. 3. Methylene-blue reduction time at 37° C.

of daily runs are shown separately for the summer and winter periods. Referring to Fig. 1 (plate counts at 37° C.), it will be seen that at Factory C (which has been shown in Table I to produce powder of uniformly good quality) there is no difference in the range or distribution of the counts between summer and winter. On the other hand, at Factories D and E (which have been shown to produce powder of relatively poor quality) the distribution of the counts varies between summer and winter, the summer period having a markedly higher proportion of high counts. Similar results are shown in Fig. 2 (plate counts at 55° C.), while from Fig. 3 it will be seen that the distribution of methylene-blue reduction times shows corresponding variations, there being no difference between the summer and winter tests for Factory C, but marked differences for Factories D and E. These results show once again that with consistent management of the plant a factory can produce milk powder of high quality throughout the year, while with poor management special difficulties will inevitably be experienced under summer conditions when not only the total bacterial content of the incoming raw milk but its content of heat resistant streptococci (16) will be exceptionally high.

Note on Figs. 1, 2 *and* 3. In the above figures the winter period was taken as extending from the beginning of October until the end of March, and the summer period consisted of the other 6 months of the year.

Each of the values plotted in the figures represents the mean value of all the samples tested from one particular day's operation at the three different factories, and they were usually the mean values calculated from three to six samples.

The plate counts at 37 and at 55° C. are given as logarithmic values and the reduction time at 37° C. in hours. The total number of samples on which the mean values for the histograms were based is given below:

	Winter Total no. of samples examined by			Summer Total no. of samples examined by		
Factory	Plate count at 37° C.	Plate count at 55° C.	Reduction test at 37° C.	Plate count at 37° C.	Plate count at 55° C.	Reduction test at 37° C.
C	30	30	29	21	21	21
D	22	22	22	39	38	39
E	18	16	18	35	17	35

(5) *Variation between full-cream and skimmed-milk powders.*

Since only a limited number of full-cream milk powder samples was examined, and since the variations in the quality of samples obtained from individual factories have been shown to be large, a direct comparison between the bacterial content of full-cream and separated-milk powders has been limited to samples obtained from a single factory, i.e. Factory E. The relevant figures are shown in Table X. It will be seen that the mean values of the plate counts differed appreciably, that of the skimmed-milk powder being about eight times as high as that of the full-cream product. Corresponding results were obtained for the methylene-blue reduction tests. A careful study of the standard errors

Table X. *Factory E*

	Powder made from	
	Whole milk	Skimmed milk
No. of samples examined	42*	107
Plate count at 37° C.:		
Mean	511,000	4,180,000
Range: Min.	1,400	5,000
Max.	45,500,000	36,000,000
Log. of plate count at 37° C.:		
Mean	5·31	6·00
Standard deviation	1·278	0·844
Coefficient of variation	24·07%	14·07%
Range: Min.	3·15	3·70
Max.	7·66	7·56
Methylene-blue reduction time at 37° C.:		
Mean	9·20	7·45
Standard deviation	1·712	1·597
Coefficient of variation	18·6%	21·5%
Range: Min.	6·0	3·5
Max.	12·5	12·5

* 11 of these samples were not included in the total for Factory E given in Table I since they were examined after the statistical analysis had been made.

of the logarithms of the plate counts indicates, moreover, that, although the ranges of the counts did not differ materially, the means are probably significantly different. The same conclusion applies to the reduction tests, and it will be seen that in this case the skimmed-milk powder actually had a wider range of reduction times, the minimum being 3½ hr. as compared with 6 hr. for the full-cream powder. It is possible that these differences are associated with variations in the quality of the incoming milk, since it might be expected that fresh whole milk would have a smaller bacterial flora than separated milk.

(6) *Variations during storage.*

(*a*) *Variations at room temperature.* Previous workers (8,10) have shown that during prolonged storage the viable bacteria initially present in milk powders tend to die out. This observation was confirmed in the present study. The following table gives a summary of the results obtained in the periodic examination of a series of samples of typical spray-dried powders:

	Time stored at laboratory temperature in months					
	0	1½	3	6	8	12
Mean % reduction in count	—	50	67	73	76	95
Range of % reduction in count	—	1–96	25–91	5–99	29–98	91–98
No. of samples examined	43	12	18	28	16	7

While the extent of the reduction in bacterial flora varied widely from sample to sample (as shown from the figures of the range of percentage reductions), the mean figures showed that there was a progressive fall during storage, plate counts made on powders kept in storage for 12 months giving counts

which were less than 10% of their initial values. Thus (to take two typical samples) with sample D 72 an initial count of 330,000 per g. was reduced to 29,000 per g., while with sample E 140 an initial count of 5,200,000 per g. was reduced to 500,000 per g.

Other tests for bacteriological quality also indicated that the number of bacteria decreased on storage. Thus with the keeping quality tests, the number of days before the samples clotted on boiling tended to increase by ½–1 day on 12 months' storage, particularly if the initial keeping quality was 3 or less than 3 days. When, however, the original keeping quality was 3½–4 days, as with many of the samples which had a low plate count, the initial keeping quality did not increase appreciably on storage.

The reduction tests at 37° C. also generally reflected a reduction in bacteriological activity as the samples aged and the reduction times increased, especially when the original times were short. For instance, the methylene-blue reduction time of sample E 26 was 3½ hr. initially; after 6 weeks' storage it had risen by 1 hr., and after 6 months it required twice as long for the methylene blue to be reduced. However, with sample F 5, the increase was less marked, the reduction time increasing to 10½ hr. from 9½ hr. after 6 months' storage.

The plate counts and reduction times at 55° C. indicated on the whole a reduction in numbers and bacteriological activity, but the results were less marked and less consistent than for the same tests carried out at 37° C.

It may be noted that this apparent improvement in the bacteriological quality of milk powders during storage is of considerable importance in relation to grading standards, since it is quite probable that a powder which failed to attain a given standard when examined soon after production would qualify by the same standard when examined after several months' storage. It is clear, therefore, that in formulating quality standards the approximate age of the powder at the time of testing would need to be stipulated.

(*b*) *Variations at different temperatures.* Table XI shows that the bacterial flora tend to die out more rapidly as the temperature of storage is raised. The initial plate counts of the powders show considerable variations (probably ranging from 100 to 250 millions per g. of powder), but if the lower value be taken as a standard it will be seen that after 6 months' storage in a refrigerator the count has been reduced by 13% (i.e. to 87,000,000 per g.), whereas at room temperature it has been reduced by 82%, at 22° C. by 98%, and at 40° C. by 99·9% (i.e. to only 94,000 per g.). Results obtained with the methylene-blue reduction tests confirmed in general these findings, the reduction times for samples stored in a refrigerator and at room temperature remaining more or less constant, those for samples stored at 22° C. rising from 6½ to 8½ hr., and those for samples stored at 40° C. rising from 6½ to 10½ hr.

(*c*) *Variations with storage in air versus nitrogen.* Opportunity was taken to determine the relative effects of storage in air with storage in an inert gas, namely nitrogen. In both cases the milk powder was packed in sealed containers, but with one set of samples the container had been exhausted of air and refilled

with commercial nitrogen.[1] An examination of Table XI indicates that in general the rate of reduction in bacterial flora was considerably retarded by the substitution of nitrogen for air. Thus after 6 months' storage at room temperature the plate count of powder in the air-filled containers showed an 82% reduction while that of powder in the nitrogen-filled containers showed only a 38% reduction. The equivalent figures after six months' storage at 22° C. were 98 and 92%. At 40° C. there were no significant differences after prolonged storage (i.e. after 6 months), since the counts of both sets of samples had been reduced by over 99·9%, but the differences were clearly apparent at the earlier stages of storage (up to 28 days).

It may be noted that the above differences in the rate of reduction of bacterial flora were seldom accompanied by any perceptible differences in the methylene-blue reduction times.

V. *The use of the methylene-blue reduction test as a method of assessing the bacteriological quality of milk powders*

Evidence has been presented in an earlier publication(17) regarding the relationship between the plate count, the methylene-blue reduction test and the keeping quality of samples of raw milk produced under a variety of conditions, and the conclusion was drawn that for the general purpose of classifying milk according to bacteriological quality the methylene-blue reduction test appears to have outstanding advantages, among which may be mentioned simplicity, rapidity and cheapness. It seemed desirable to make a similar comparison of the relative values of the plate count and the reduction test in assessing the quality of milk powders.

Owing to the relatively small range of variation in the keeping qualities of the majority of samples examined[2] it was not practicable to attempt to correlate either of the above tests with keeping quality. The comparison has, therefore, been limited to a direct correlation between plate count and reduction test. Full data for the tests carried out at 37° C. are given in the form of a frequency table in Table XII. It will be seen that the majority of the figures fall along a broad band, the discrepancies being somewhat more numerous than with samples of raw milk(17). This difference is reflected in the correlation coefficients for the two series, the value of r for the dried-milk samples being $-0{\cdot}723$ as against $-0{\cdot}818$ for the raw-milk samples.

It may be noted, too, that the regression equations for the two series differ, as shown in Table XIII.

[1] The chief object of using nitrogen was to attempt to prevent or retard fat deterioration (tallowiness), which is recognized to be due to oxidation. The bacteriological studies were therefore incidental to the main object of the investigation.

[2] It will be seen on reference to Table III that 93% of the samples had keeping qualities of between 3 and 4 days, which would allow only three values for comparison, i.e. 3, 3½ and 4 days. It was felt that this would be quite inadequate to enable a valid correlation coefficient to be calculated.

Table XI. *Storage temperature*

No. of days storage	Refrigerator (approx. 4° C.) plate count at 37° C.		Room temperature (approx. 10° C.) plate count at 37° C.		22° C. plate count at 37° C.		40° C. plate count at 37° C.	
	Tins air filled	Tins nitrogen filled	Tins air filled	Tins nitrogen filled	Tins air filled	Tins nitrogen filled	Tins air filled	Tins nitrogen filled
9	111,000,000	70,000,000	141,000,000	81,000,000	59,000,000	154,000,000	33,000,000	60,000,000
18	147,700,000	102,400,000	54,400,000	89,400,000	81,500,000	126,200,000	2,730,000	11,200,000
28	93,000,000	265,000,000	88,000,000	102,000,000	51,000,000	98,000,000	1,570,000	4,600,000
84	88,000,000	181,000,000	89,000,000	97,000,000	16,200,000	28,300,000	330,000	490,000
127	148,000,000	160,000,000	20,600,000	86,000,000	7,600,000	13,100,000	173,000	231,000
140	103,000,000	175,000,000	204,000,000	302,000,000	7,900,000	12,400,000	260,000	289,000
164	88,000,000	77,000,000	39,000,000	65,000,000	4,400,000	18,500,000	112,000	160,000
185	87,000,000	57,000,000	18,500,000	62,000,000	2,520,000	7,900,000	94,000	79,000

Table XII. *Frequency table*

Log of the plate count at 37° C.	Methylene-blue reduction time in half-hours at 37° C.																						
	3	3½	4	4½	5	5½	6	6½	7	7½	8	8½	9	9½	10	10½	11	11½	12	12½	13	13½	14	Total
3·01–3·25	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	1	.	.	.	2
3·26–3·50	.	.	.	.	.	.	.	.	.	.	.	.	.	1	1	.	.	.	.	2	.	2	.	6
3·51–3·75	.	.	.	.	.	.	1	.	.	.	.	.	1	.	.	.	4	3	1	1	2	.	.	13
3·76–4·00	.	.	.	.	.	.	.	.	.	.	.	.	.	2	3	2	3	1	2	1	3	.	1	18
4·01–4·25	.	.	.	.	.	.	.	.	.	.	1	1	1	1	2	.	3	2	1	1	.	1	.	14
4·26–4·50	.	.	.	.	.	.	1	.	.	2	.	.	3	4	4	1	3	.	1	.	.	.	1	20
4·51–4·75	.	.	.	.	.	.	.	.	.	1	3	2	5	2	3	1	2	.	.	.	.	.	.	19
4·76–5·00	.	.	.	.	.	2	.	1	1	1	1	4	2	5	.	1	.	.	1	.	.	.	.	19
5·01–5·25	.	.	.	.	.	.	.	1	2	5	4	3	5	6	1	1	.	.	.	.	.	.	.	28
5·26–5·50	.	.	.	.	1	1	.	.	1	4	2	3	4	1	.	1	.	.	.	1	.	.	.	19
5·51–5·75	.	.	.	.	.	2	.	.	5	5	2	10	.	1	1	2	.	.	1	.	.	.	.	29
5·76–6·00	.	.	.	.	1	1	1	5	11	7	3	6	3	1	1	2	.	.	.	.	.	.	.	42
6·01–6·25	.	.	.	.	.	3	2	9	6	7	3	3	3	1	1	.	1	.	.	.	.	.	.	39
6·26–6·50	.	.	.	.	3	3	9	8	6	7	2	3	6	2	1	.	.	.	.	.	.	.	.	50
6·51–6·75	.	.	.	1	2	2	2	5	2	3	1	.	1	.	.	.	.	.	.	.	.	.	.	19
6·76–7·00	.	.	.	.	.	5	1	1	4	5	.	3	1	1	.	.	1	.	.	.	.	.	.	22
7·01–7·25	.	.	.	2	.	.	.	2	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	5
7·26–7·50	.	1	.	3	.	1	1	2	.	2	.	.	.	.	.	.	.	.	.	.	.	.	.	10
7·51–7·75	.	.	.	1	.	1	.	1	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	4
7·76–8·00	1	.	.	.	.	.	.	3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	4
8·01–8·25	1	.	.	.	.	1	1	2	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	6
	2	1	.	7	7	22	19	40	40	49	23	38	35	28	18	11	17	6	8	7	5	3	2	388

Table XIII

	Regression equations	Value of y when plate count is 200,000 per ml., i.e. when $x = \log 5{\cdot}30$ hr. min.	Value of x when $y = 5{\cdot}0$ hr.	Plate count per ml. equivalent to value in preceding column
Raw milk samples (17)	$x = -0{\cdot}269\,y + 6{\cdot}514$ $y = -2{\cdot}486\,x + 18{\cdot}17$	4 59	5·17	148,000
Dried milk samples	$x = -0{\cdot}3877\,y + 8{\cdot}778$ $y = -1{\cdot}349\,x + 15{\cdot}769$	7 16	5·84*	690,000*

x = logarithm of the plate count, y = time in hours required to reduce methylene-blue in the reduction test.

* These figures refer to the reconstituted milk powder, which will contain per ml. only one-tenth of the bacterial flora which is present *per g.* of powder.

From this table it will be seen that for a given plate count the bacterial flora of reconstituted milk powder show a less active reducing power than the flora of raw milk; e.g. when the count is 200,000 per ml., the equivalent reduction time for reconstituted milk powder is $7\frac{1}{4}$ hr. compared with 5 hr. for the raw milk. It seems clear that this must be due to the very specialized type of flora which (as has already been shown) normally occurs in milk powders.

Since part of this flora consists of thermophilic and thermoduric bacteria, it was felt that a better correlation might exist between plate counts and reduction times when these were carried out at 55° C. Table XIV shows, however, that the frequencies are widely scattered, and calculation of the correlation coefficient gives the low value for r of $-0{\cdot}221$.

It appears from the above facts that when the reduction test is carried out at 37° C. it shows reasonable agreement with the plate count *provided that the correct regression equation is used.* On the other hand, there is a lack of agreement between the reduction test at 55° C. and the numbers of thermophilic and thermoduric bacteria in the milk powder. This fact does not, however, invalidate the conclusion already stated on p. 205 that the reduction test at 55° C. can provide useful information regarding the activity of such organisms.

VI. *A note on the desirability of grading spray-dried milk powder*

Data have been presented which show that wide variations exist in the bacteriological quality of spray-dried milk powder, but that these variations are least in the powder from the factories which generally produce the best quality powder. It has been indicated that powders of high bacteriological content are probably due to poor technique of operation at the factory. Hucker & Hucker (18) have also demonstrated this fact and have shown that by the introduction of special sanitary methods at drying plants the counts of commercially prepared infant foods were greatly reduced. Hence it seems reasonable to suppose that milk powder of high bacteriological quality can be consistently produced.

The use of milk powder in the preparation of other food products is becoming of increasing importance, but it is essential to the manufacturer of a

Table XIV. *Frequency table*

Log of the plate count at 55° C.	Methylene-blue reduction test at 55° C. (in half-hours)																									Total
	3½	4	4½	5	5½	6	6½	7	7½	8	8½	9	9½	10	10½	11	11½	12	12½	13	13½	14	14½			
1·76–2·00	.	.	.	.	.	.	1	1	.	.	.	.	.	.	1	.	.	1	.	.	.	.	.			4
2·01–2·25	.	.	.	.	.	1	.	.	2	1	.	.	.	1	.	.	.	.	.	.	1	.	.			7
2·26–2·50	.	.	.	.	.	1	.	1	.	1	1	.	2	1	.	.	.	.	.	.	.	.	.			7
2·51–2·75	.	.	.	.	.	.	1	1	.	.	1	5	1	2	1	.	1	.	1	.	.	.	.			14
2·76–3·00	.	.	.	.	.	1	.	.	3	.	.	.	2	.	2	2	1	1	2	2	2	.	1			19
3·01–3·25	.	.	.	.	.	.	.	1	4	2	2	1	2	1	2	1	.	.	.	2	.	.	.			16
3·26–3·50	.	.	.	1	.	.	.	2	1	1	3	2	2	5	2	1	1	.	1	.	.	.	.			25
3·51–3·75	.	.	.	.	.	.	.	.	1	1	1	2	1	1	4	.	.	1	1	3	2	1	.			19
3·76–4·00	.	.	.	.	.	3	.	1	1	1	2	3	3	2	.	3	2	2	1	1	1	.	.			26
4·01–4·25	.	.	.	.	1	.	.	.	1	4	1	1	1	.	.	1	.	.	.	2	.	.	.			12
4·26–4·50	2	.	.	.	1	.	.	2	1	5	2	1	.	1	3	.	1	.	.	.	.	1	.			22
4·51–4·75	.	.	.	.	.	1	1	5	2	4	2	2	1	3	2	1	1	1	1	.	.	.	.			27
4·76–5·00	.	.	.	1	1	.	.	3	2	2	.	1	1	1	1	2	.	.	.	.	.	.	.			16
5·01–5·25	.	.	1	.	.	2	4	1	2	1	1	1	.	1	.	.	.	.	.	.	.	1	.			15
5·26–5·50	.	.	.	.	.	1	.	1	1	4	.	1	.	1	2	.	.	.	.	.	.	.	.			11
5·51–5·75	.	.	.	.	.	1	1	.	1	1	1	3	1	2	.	.	.	.	.	.	.	.	.			11
5·76–6·00	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.			1
6·01–6·25	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.			2
	2	.	1	2	3	11	11	18	22	27	20	23	17	22	20	10	8	6	8	9	6	7	1			254

proprietary food product that he should maintain a uniform quality in the product from day to day. In order to do this he obviously requires ingredients of unvarying quality. It is clear therefore that the grading of milk powders would be of value to the purchaser. Moreover, the provision of definite grade standards of quality would also act as an inducement to raise the standards of operation at many existing milk-drying plants. The value of such grading has already been appreciated in the United States where the American Dry Milk Institute(4,19) has successfully operated grades for skimmed-milk powder since 1930.

It is only intended here to mention briefly some of the factors which might affect the bacteriological standards for milk powders. Bacteriological grades should probably be based entirely on the examination of the final product, but as a general safeguard the incoming milk, which should be fresh, clean and free from abnormal taste and smell, must be pasteurized, preferably by the holder system, at a minimum temperature of 145° F. for at least half an hour. It might in some operations be simpler to "flash" pasteurize the milk, but the process must be controlled so that the incoming raw milk or partially heated milk is not mixed with that which has been rendered free from disease-producing organisms by pasteurization. Moreover, from a chemical point of view the heat treatment should be sufficient to destroy any enzymes (e.g. lipase) which might adversely affect the keeping quality of the powder.

It would be important, if grades were adopted for spray-dried milk powder, to specify that all the testing should be carried out within a definite time after manufacture, since ageing seriously changes the quality. Three grades, namely, First, Standard and Third Grade, are in operation in the United States for dried-milk solids, and the plate count at 37° C. forms the basis of the bacteriological limits of the grades. Similar standards might well be adopted in this country. In order to determine what percentage of the samples would fall within the limits of the plate count specified by the American grades and within the limits of the same count expressed in terms of the reduction test, the results of the tests carried out during the present survey have been collected in Table XV.

It will be seen from this table that 36% of the samples of spray-dried milk powder would fall within the American First Grade, and 48% within the Standard Grade, while over 40% would be classed as Third Grade. If the reduction times corresponding to the plate counts which have been obtained from the regression equation of these samples were to be used instead of the plate counts, 36% of the samples would be classed as First Grade, 54% as Standard and 48% as Third Grade. It would appear, therefore, that the reduction test, if used alone for grading, would impose a slightly more severe standard and that a reduction time of 8 hr. or more might be taken to indicate a powder of relatively good bacteriological quality.

Table XV

American Dry Milk Institute grade designation	Bacterial count per c.c. of reconstituted milk for grade	Log of count of preceding column calculated per g. of powder	Reduction times equivalent to log of plate count of preceding column hr.	Reduction time taken as hr.	Percentage of samples within the plate count standard of grade	Percentage of samples within the reduction test equivalent to plate count of grade
First	15,000	5·18	8·78	9	36	36
Standard	50,000	5·70	8·09	8	48	54
					Percentage of samples over the plate count which is lower limit for grade	Percentage of samples over the reduction test equivalent to plate count of preceding column
Third	Over 100,000	6·00	7·68	7½	41	48

Summary

1. Over 400 samples of spray-dried milk powder from eight factories operating in England and Scotland have been examined. These samples were taken at different stages of the day's run and were obtained from several types of plant situated in widely different districts. Samples were taken in both the summer and winter months. The samples were, therefore, sufficiently representative to enable general conclusions to be drawn regarding the factors affecting bacteriological quality.

2. In reconstituting milk powders for bacteriological examination it is necessary to ensure (i) that the sample is sufficiently large to be representative, and (ii) that external contamination is avoided during weighing and handling. The use of a 20 g. sample is advocated. Complete reconstitution of the powder is best obtained by shaking the sample with glass beads in a 180 ml. water blank which is contained in a screw-capped bottle. Details are also given of the methods used in bacteriological examination of the samples and for the isolation of pure cultures from them.

3. From an analysis of the results the arithmetic "weighted" means of the plate count and methylene-blue reduction test at 37° C. were found to be 4,363,000 per g. and 8·1 hr. respectively. Individual plate counts varied widely, viz. from 1400 to 149,000,000 per g., whilst the reduction tests varied from 3 to 14 hr. The plate counts at 55° C. also showed variations, although the range was smaller than at 37° C. The reduction times were longer at 55° C. than at 37° C., and 30% of the samples required more than 12 hr. incubation before decolorization occurred.

4. From a detailed study of the results, information has been obtained regarding some of the factors responsible for these variations in bacterial quality. Wide variations in the mean of the plate counts of powders have been shown to

occur from one factory to another, and this appeared to be mainly associated with the technique of manufacture. It was also demonstrated that, since there was a marked difference in the range of variations of samples obtained from individual factories, there must have been a complete lack of consistency in the day-to-day management of certain of the plants. Some evidence is presented which tends to show that the bacteriological quality of milk powders may usually deteriorate during a continuous day's run owing to the multiplication of thermophilic and thermoduric bacteria during processing.

An examination of the data further demonstrated that seasonal variations in quality only occurred in plants where the general bacterial standard of the powder was normally poor. A comparison between a limited number of full-cream milk powder samples and skimmed-milk powder samples made at one factory indicated that the full-cream powders were usually of a higher bacteriological quality. It is possible that these differences may have been associated with variations in the quality of the incoming milk, since fresh whole milk might be expected to have the smallest bacterial flora. During prolonged storage the viable bacteria which were present in the milk powders died out. The mean percentage reduction in count after 12 months' ageing at room temperature was 95%. The bacterial flora tended to die out more quickly as the temperature of storage increased and decreased faster when stored in an atmosphere of air than in one of nitrogen.

5. The fermentation test has been shown to be mainly useful as an indication of the activity of thermophilic bacteria at 55° C.

6. About 10% of the samples tested in 1 ml. quantities gave positive presumptive coliform tests, but some of the positive tests were due to anaerobic sporeformers.

7. The number of yeasts and moulds present in the milk-powder samples was found to be practically negligible.

8. The keeping quality of the reconstituted milk when stored at 60° F. has been examined. Over 90% of the samples remained sweet for between $2\frac{1}{2}$ and $3\frac{1}{2}$ days. Tests made on these samples after 24 hr. storage indicated that the flora of reconstituted milk did not increase as rapidly as that of raw milk, and that the reduction test was the most sensitive indication of the additional ageing of the milk.

9. The predominating flora of the reconstituted milk at 37° C. rendered it acid in reaction and clotted it. 80% of the 1304 cultures picked off the plates incubated at 37° C. and 74% from the plates held at 55° C. produced acidity and coagulation in litmus-milk media.

10. More than 97% of the pure cultures obtained from colonies on the plates incubated at 37° C. which produced acid coagulation of milk were found to be heat-resistant streptococci. Further classification showed that 66% were strains of the "*viridans*" division, 57% being *Str. thermophilus* and 33% of the "*enterococcus*" division, 26·5% of which were *Str. durans*.

11. The milk-curdling organisms obtained from the plates incubated at 55° C. have been identified as strains of *B. calidolactis*, which is one of the few thermophilic sporeformers capable of producing acid coagulation of milk.

12. A few bacteria causing peptonization of milk at 37° C. have also been classified, and these were all found to be aerobic sporeformers of the *B. subtilis* type.

13. The use of the methylene-blue reduction test as a method of assessing the bacteriological quality of milk powders has been explored and the conclusion is reached that this test gives reasonable agreement with the plate count at 37° C. provided that the correct regression equation is used. The reduction test at 55° C. does not appear, however, to be as useful as that at 37° C.

14. The desirability of establishing grades for spray-dried milk powder is discussed.

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223. BACTERIOLOGICAL STUDIES OF CANNED MILK PRODUCTS

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In the present investigation of the bacteriology of canned milk products the greater part of the work has been concerned with evaporated milk and canned cream. These two products have been considered together because, since their methods of manufacture are similar, it might be expected that bacteriological defects in evaporated milk which have been reported in the past might be encountered also in canned cream. It was felt also that a study of canned cream might be of special value because it is a relatively recent development of the canning industry and information regarding the bacteriological side is scanty.

I. EVAPORATED MILK AND CANNED CREAM

Previous investigations (see reviews 1 and 2), nearly all of which are confined to the products of one particular factory usually in connexion with a specific outbreak, indicate that although some workers invariably found evaporated milk to be sterile, others have occasionally found living organisms in the cans even when the contents have appeared to be sound. A few workers have also tested a general selection of samples with similar conflicting results. Thus while Weinzirl (1) investigated twenty-three market samples of evaporated milk, none of which revealed the presence of any living organisms, Park *et al.* (1), examining a general collection of thirty-seven tins from nineteen brands, found that they all contained living bacteria. Savage & Hunwicke (1) tested twenty-seven shop samples, consisting of different brands, together with seventeen tins obtained from three different factories, 18% of which were unsterile.

General investigation

A general survey of a large number of samples of evaporated milk and canned cream produced by three condensed milk factories, designated *X*, *Y* and *Z*, operating in the west of Scotland, was undertaken to determine to what extent non-sterility occurred in commercial practice and what organisms were present after processing. Information was also desired as to the relative importance of different defects and their associated organisms. Samples were obtained at about weekly intervals for nearly 2 years, from two to eight samples being examined from each individual batch.

A further number of tins was tested over a period of 4 years from other sources of manufacture. These samples were purchased from shops or obtained

directly from other factories, making a total of 315 evaporated milk samples and 253 samples of canned cream. In addition to the above samples which were bought as sound, many samples have been examined which were either obviously defective from the apparent bulging of the cans themselves or from the "shake sound", or were sent in by firms as tins from a batch similar to that in which defective cans had occurred. About 150 of these special evaporated milk samples and 115 special samples of canned cream have been tested.

Experimental technique

(1) *Can opening*

Samples from the factories were first incubated at 37° C. for varying periods of time; usually they had at least 6 days' but not more than 21 days' incubation. The remaining samples were also incubated before testing unless they were obviously faulty or old when they were opened at once. Several methods of sampling the contents were tried, since the opening of cans and the removal of part of the contents without aerial contamination presented obvious difficulties, especially if the can was distended by gas. Finally the following technique was adopted. The top of the can was washed thoroughly and formalin rubbed over it. About 5 ml. of absolute alcohol were then ignited on the surface and a Bunsen burner held so that the flame played on the can top. A hole, up to $\frac{1}{2}$ in. in diameter, was made by a "cold" chisel, which had previously been heat sterilized, and the tin was immediately covered by a sterile Petri dish lid.

(2) *Culturing*

The culturing technique which was found to be most suitable was the heavy inoculation of the product into litmus milk, nutrient broth and dextrose broth. The inoculated media were then incubated at 37° C. for at least 1 week. The nutrient broth was prepared from 0·5% peptone, 0·3% marmite (or occasionally lemco), and tap water, but glucose broth had in addition 2% glucose which was added before the final sterilization. The final *p*H of the broths was about 6·8.

The inoculum was removed from the tin by a 1 ml. pipette, the tip of which had been widened to allow for the viscous nature of the product. At least 1 ml. was used, since it was found that if smaller inocula were employed the numbers of organisms present in the can were sometimes so small that they were not recovered. If the product was obviously spoiled but the presence of bacteria had not been demonstrated by the above technique, the inoculated media were further incubated at 55° C., in order to test for the presence of thermophilic organisms. Cooked meat media and dextrose-agar shakes were inoculated from some of the samples to determine whether anaerobes were present, and occasionally inoculations were made into these media from the media already incubated at 37° C. if no other organisms had been recovered. At the beginning of the investigation agar plates were streaked with the product directly, and in addition plate counts were made to give an indication of the number of bacteria present. Plating was found, however, to be of little value and is not recommended. Esty & Stevenson (3), in similar examinations of canned foods, also abandoned direct plating as being of doubtful value. Films for microscopic examination were made directly from the product and have given useful indications of the flora, but when the numbers are low, as in old samples, the apparent absence of organisms on microscopic examination may be misleading.

(3) *Can contents*

The colour, odour, consistency and general condition of defective samples of evaporated milk and canned cream were noted at the time of testing so that it might be possible to determine whether specific defects were associated with certain types of bacteria.

(4) *Recovery of bacteria from unsterile cans and their identification*

When possible, the organisms in the unsterile samples were purified by dilution and plate culture, and were further examined for morphological, cultural and biochemical characteristics. A list of tests used for the differentiation of the isolated bacteria is given below.

Type of cell and colony.
Motility.
Staining properties: Gram and with common dyes.
Spore formation.
Growth on agar slant,
in agar stab,
in glucose agar stab,
in gelatine stab at 22° C.
Growth in litmus milk,
in broth,
in glucose broth,
in MacConkey's bile salt broth.
Final *p*H in glucose broth.
Carbohydrate fermentations: levulose, glucose, lactose, maltose, raffinose, salicin, mannite, starch.
Nitrate reduction: 1% KNO_3.
Indol production: Ehrlich's method.
Voges-Proskauer test: Barritt's modification.
Reduction of methylene blue: 0·1% in milk.
Splitting of sodium hippurate.
Splitting of aesculin.

As the composition of the media used did not differ appreciably from the normal no details have been given. All the organisms were not examined by every test; a decision as to which tests were employed depended on whether the cultures were heat resistant. The incubation temperature was usually 37° C.

RESULTS AND DISCUSSION

(1) *Sterility of commercial samples*

(*a*) *Evaporated milk.*

100% of the tins which had been obtained from general sources were found to be sterile. It is probable that the lower value of the factory samples, namely 94 and 93% (Table I), would be associated with some special circumstance of operation. With the samples from factory *X* definite evidence of this was available, as more than half of the unsterile samples from this factory were produced during a 10-day period, when it seemed likely that the processing was not being carried out as efficiently as usual.

Tests on the special samples which were examined because they were either obviously defective or associated with defective packs showed, as would be expected, a much higher percentage to be unsterile.

(*b*) *Canned cream.*

100% of the samples tested from general sources were again found to be sterile, but the percentage of sterile factory samples was markedly lower, being only 85 and 76%. In seeking an explanation of this it was found that the

percentage of unsterile factory samples was considerably higher with 12 oz. cans than with smaller packs. The smaller containers examined consisted of 3 and 6 oz. can samples, together with a few small glass jars. It might be anticipated that it would be more difficult to sterilize the large cans, since there would be a lag in attaining the necessary temperature compared with the smaller containers. The difficulty in adjusting the process to compensate for this lag would be particularly marked where the factory had been unaccustomed to process 12 oz. cans, and inquiry revealed that the majority of these samples were examined when the factories were processing the large cans for the first time. The figures given in Table II show clearly that the 12 oz. cans were chiefly responsible for the high non-sterility figures. 20% of the total cans of cream examined were unsterile, but the corresponding percentage for 12 oz. cans was 77, while for the small packs it was only 8. It will be seen that unsterile large tins accounted for 65% of the total non-sterile canned cream figures.

(2) *Types of defective samples encountered and associated bacteriological flora*

Table III summarizes the types of defective samples which were investigated and the associated bacteria isolated from them. It should be noted that in the classification into different defects the line of demarcation was not always so clear as would appear from the table, although it is probably true that the type of defect might have developed further on prolonged storage.

By using the resistance to heat of the organisms which were recovered as a broad basis of classification, the defects have been divided into two groups, under which they will be considered. The two groups are: (A) a widely mixed general flora containing varieties of organisms which do not withstand high temperature, and (B) a restricted flora which is heat resistant and of the sporeforming type.

The reasons for differentiating the organisms into these two groups may be briefly stated as follows: Non-heat-resistant bacteria will almost invariably gain access to the can during the cooling process when, through bad seaming or faulty sealing, leakages of air and water into the can may occur. Such contamination may therefore be looked upon as accidental. Heat-resistant bacteria will, however, usually be present as a result of inadequate sterilization. There will of course be exceptions to these two generalizations, e.g. gross understerilization may result in the survival of non-heat-resistant types, while sporeformers might be among the organisms which enter accidentally through mechanical defects in the cans.

A. Spoilage due to non-sporeformers

In order to divide the organisms isolated from defective samples into one of the two main groups a study was made of their heat resistance. Small tubes of sterile skim milk were inoculated with the cultures and then heated for 20 min. at different temperatures in a thermostatically controlled glycerine bath. It

Table I. *Sterility of commercial samples*

Product tested	Source	No. of samples examined	No. of batches from which samples obtained	No. of sterile samples	% of sterile samples	No. of samples not sterile	No. of batches from which unsterile samples obtained	% of unsterile samples
Evaporated milk	Factory *X*	235	125	222	94	13	10	6
	,, *Y*	44	26	41	93	3	2	7
	,, *Z*	1	1	1	100	0	—	0
	General sources, shops, etc.	35	30	35	100	0	—	0
	Total	315	182	299	95	16	12	5
	Special samples faulty?	152	52	93	61	59	22	39
Canned cream	Factory *X*	52	22	44	85	8	4	15
	,, *Y*	6	3	6	100	0	—	0
	,, *Z*	178	82	136	76	42	24	24
	General sources, shops, etc.	17	17	17	100	0	—	0
	Total	253	124	203	80	50	—	20
	Special samples faulty?	115	49	80	70	35	14	30

Table II

Source	No. of samples examined		No. of sterile samples		% of sterile samples			No. of unsterile samples		% of unsterile samples			% of total non-sterile samples accounted for by 12 oz. cans
	Small containers	12 oz. cans	Small containers	12 oz. cans	Small containers	12 oz. cans	Total of all containers	Small containers	12 oz. cans	Small containers	12 oz. cans	Total of all containers	
Factory *X*	43	9	40	4	93	44	85	3	5	7	56	15	10
,, *Y*	6	0	6	0	100	0	100	0	0	0	0	0	0
,, *Z*	143	35	130	6	91	17	76	13	29	9	83	24	16
General sources, shops, etc.	17	0	17	0	100	0	100	0	0	0	0	0	0
Total	209	44	193	10	92	23	80	16	34	8	77	20	13

Table III. *Defective samples and associated micro-organisms*

Source	No. of unsterile samples	Type of defect: "Bloats" usually with coagulation — Mixed flora	Coagulation — Non-sporeformers	Coagulation lumpy. Mixed flora sporeformers and non-sporeformers	Soft coagulation, some also putrid flavour — Sporeformers	Putrefactive odour — Sporeformer	Putrid flavour, bitter — Thick — Sporeformers	Putrid flavour, bitter — Thin — Sporeformers	"Bloat" — Defect appearing to be bacteriological but no organisms isolated	Coagulation — Defect appearing to be bacteriological but no organisms isolated	Product free from defect — Sporeformers present	Product free from defect — Anaerobic sporeformers present	Product free from defect — Mixed flora non-sporeformers present	No details of defect or organisms retained from these unsterile samples
Evaporated milk														
Total samples from factories and general sources	16	3	—	—	7	—	—	—	—	2	1	—	1	2
Special	59	30	12	1	3	1	1	—	7	—	—	—	4	—
Canned cream														
Total samples from factories and general sources	50	2	1	—	6	—	1	—	—	—	35	2	—	3
Special	35	4	1	—	3	—	2	22	1	1	1	—	—	—

was found that nearly all the non-sporeformers were killed at from 60 to 65° C., except for a few cocci which required a temperature of 69–70° C. Thus it is clear that, if they did not gain entrance to the cans after processing, the latter must have been grossly understerilized.

It is doubtful whether any importance can be attached to the actual type of non-heat-resistant organisms present in defective cans, since, if contamination is due to mechanical defects, the flora entering the can is likely to be mixed. A diversity of type of defect and of causative bacteria is therefore inevitable. Moreover, even if defects associated with non-heat-resistant organisms were due to serious understerilization, a fairly large variety of types, probably chiefly thermoduric streptococci, would survive. It is probable that in an excellent medium like milk most of the contaminating organisms would find conditions suitable. It may be noted that when faulty containers are the cause of contamination spoilage usually occurs in casual cans.

Such spoilage may be classed under two general heads as follows:

(i) *Gassy fermentation.* When this occurs the "blown" cans (or "bloats" or "swell heads" as they are usually termed) become bulged at the ends, due to the evolution of gas which is usually the result of bacterial metabolism. In extreme cases, such fermentation may be accompanied by bursting of the seams.

Savage & Hunwicke (1) found that *Bact. coli.*, *Bact. cloacae* strains, a yeast, a streptococcus, a diplococcus, and some pleomorphic coccoidal organisms were responsible for "bloats" in evaporated milk, and that in most samples the milk was also clotted. Hammer (4) reported a case of fermentation due to a gas-producing *Streptococcus*, in which the milk failed to clot, although the flavour was abnormal.

Nearly half the defective evaporated milk samples examined in the present survey (Table III) were "bloats" and gassy fermentation was the commonest single cause of defective unsweetened canned milk. As regards the condition of the contents of the affected milk, the extent to which frothing occurred after opening varied, and the consistency of the contents also varied, although nearly all the samples were clotted. The samples often had, in addition, an unpleasant sour "cheesy" smell and a bad flavour. When the product was clotted it could be detected by the "thumping sound and feel" when the tins were shaken. Canned cream "bloats" were similar in character to those described for evaporated milk, but were less numerous.

A variety of different bacteria was isolated from the "bloats", which often had a mixed flora. Cocci were the commonest types of organism isolated and many strains were recovered. Although almost all the cocci quickly produced acidity in sugar media and curdled milk they were not able to produce gas and therefore were not responsible for the "swells", although they obviously contributed to the curdling.

Of the organisms isolated, those capable of producing gas when reinoculated into canned products were entirely of the short rod, gram negative, *Escherichia* genus and related bacteria. This group of organisms ferments many carbo-

hydrates with the production of acid and gas. It rapidly curdles milk and forms a gassy curd with unpleasant odour. Sixteen cultures were examined in greater detail and were finally classified by their reactions as:

Strains of *Escherichia coli communis*	12
,, *Aerobacter lactis aerogenes*	1
,, *Aerobacter cloacae*	3

One species of yeast was also isolated, but since it did not ferment lactose it was obviously not responsible for the "swell".

(ii) *Coagulation.* Micro-organisms isolated from cans of which the contents were coagulated and sour, but in which gas production was absent, were almost entirely of the cocci type and were similar to those mentioned as isolated from "bloats".

It may be noted that several cultures of cocci have been recovered from products which did not exhibit any defect. These bacteria did not alter litmus milk or evaporated milk on prolonged incubation, although they grew on agar media. They probably gained entrance by the same channels as the other non-heat-resistant non-sporeformers.

(iii) *Other defects.* Two further types of defect, namely bitterness(5) and fishiness(6), have been attributed in the past to the non-sporeformers. Neither bitterness due to non-sporeformers nor fishiness was encountered in the present investigation.

In eleven defective samples, six of which were at least 3 months old, it was not found possible to recover viable organisms although the contents were either "bloated" or coagulated. In this connexion it may be noted that it was very difficult to keep these organisms alive for any considerable length of time in the laboratory unless special precautions were taken. It therefore seemed probable that in these eleven defective samples the causative organisms had died out during storage.

B. Spoilage due to sporeformers

Before dealing in detail with spoilage caused by sporeformers it should be observed that although such spoilage is usually more widespread than occurs with the non-heat-resistant types, the contents of many cans from an affected batch may be found to be sterile. Thus conclusions as to the sterility of any batch which are based on the examination of a single can may be misleading. Esty & Stevenson(3) have stressed this point and have suggested that a minimum of twenty-four cans should be used as a basis for diagnosis. As already noted, in this investigation up to eight cans from each batch were examined.

Anaerobic sporeformers

It has been reported by Savage & Hunwicke(1) that sporing anaerobes were responsible for unsoundness in three out of forty-seven samples, and that

anaerobic sporeformers could be present in sound samples also. Hunziker(7) reported epidemics of gassy fermentation which involved a number of cans which he found to be due to *Plectridium foetidum*. This obligate anaerobe had a thermal death-point between 245 and 250° F., and nearly always produced a foul odour and intense putrefaction.

In the present study over eighty sporeformers have been isolated but only two were anaerobic, and these were obtained from sound cans. The number of anaerobic sporeformers which normally survive processing and which cause defects in the products cannot therefore be of great importance.

Aerobic or facultative aerobic sporeformers

In nearly all instances where aerobic or facultative aerobic sporeformers have been isolated only one species has been obtained from each batch of product where spoilage has been evident. The condition of the milk or cream in such cans has varied from a smooth, soft clot with or without lumpiness to a very thin consistency, the smell and flavour being putrid, bitter, or slightly indicative of protein breakdown. All combinations of consistency and off-flavour have been found, in spite of the fact that in the samples examined the bacteria isolated were all closely related.

(*a*) *Classification of aerobic sporeforming organisms isolated from canned cream and evaporated milk.*

Fifty-two cultures of aerobic or facultative aerobic sporeformers have been examined in detail. Included among these are some cultures which were isolated from products which were not noticeably defective when examined, although the organisms resembled very closely those isolated from faulty cans. For instance, thirty-five cream samples appeared to be sound, yet this type of sporeformer was isolated from them. These cans had, however, only been subjected to about a week's incubation at 37° C. It will be shown later that this incubation period may be insufficient to allow the full development of spoilage.

In the past there has been some confusion in the classification of this group. *B. subtilis* has been regarded as the type species, but different workers have employed various strains of this organism as a basis of classification. In 1936, however, the Second International Microbiological Conference decided that Gottheil's strain of *B. subtilis* (or the so-called Marburg strain) should be recognized as the type culture for the aerobic sporeformers. Since the type culture has been fixed, confusion has been reduced, and the classification adopted here has been that suggested by Gibson (8–10). The different strains isolated were of the same general type, i.e. they grew well on common media, spored readily and digested gelatine and litmus milk, all, except *B. circulans*, producing an

alkaline reaction in the later stages. The numbers of each strain were found to be as follows:

	No. of strains	% of total
*B. subtilis**	36	69
*B. licheniformis**	8	15
*B. pumilus**	3	6
B. brevis	2	4
B. circulans	2	4
B. cereus	1	2
Total	52	100

* These cultures have been referred to earlier (11) as *B. vulgatus*, *B. subtilis* and *B. mesentericus*. The names given here are now in agreement with the established type species.

The largest number of one species was 69% of *B. subtilis*, which together with *B. licheniformis* and *B. pumilus* are probably the most heat resistant, and occur commonly in milk, especially if the conditions of the byre are dirty. It may be noted that these organisms were found as spreaders on the 1/10 and 1/100 plates in the milk samples produced under dirty conditions which have been examined during a study of the factors affecting the bacteriological quality of raw milk (12). Thus bulk milk, unless it has a high standard of cleanliness during production, commonly contains numbers of heat resistant sporeformers, which are a potential source of spoilage in canned dairy products.

(*b*) *Spoilage induced by aerobic sporeformers.*

In order to confirm the relationship between aerobic sporeformers and the defects already mentioned, it was obviously desirable to investigate to what extent these bacteria (isolated chiefly from defective products) could reproduce spoilage on inoculation into sound cans. With this object thirty-one cultures were each inoculated into from three to six tins of evaporated milk or canned cream and incubated at 37° C. The cultures inoculated consisted of twenty-two strains of *B. subtilis*, four of *B. licheniformis*, two of *B. brevis* and *B. circulans*, and one strain of *B. cereus*. In addition some more strains of *B. subtilis* have been inoculated into canned cream only.

The following method was adopted for inoculating the cans. A small hole about $\frac{1}{8}$ in. in diameter was made in the top by the same method as was used for sampling the products. A loopful of culture was introduced into the cream or evaporated milk and the can was resealed by soldering a sterilized circular piece of tin plate about 1 in. in diameter over the hole. The cans were opened after periods of incubation of from 1 week to 18 months, and were examined in a similar manner to those opened during the general survey. Control cans were also incubated and examined during the time the work was in progress.

From such artificial reinoculation it might have been expected that a definite connexion between defective canned dairy products and the aerobic sporeformers, whether or not they were isolated from faulty or sound cans, would have been established. However a clear connexion between the two was not found to exist, as will be seen from the following summary of results.

(1) Only one outstanding defect was induced by the inoculation of the aerobic sporeformers, that of thinning and the associated bitterness of flavour.

This defect was found to be of great commercial importance, 63 % of the spoilage in the canned cream examined being of this type. It was therefore made the subject of a special study which has already been published (13).

(2) There was, however, a slow development of a slightly proteolytic, putrid, and/or astringent flavour in many of the cans of evaporated milk and cream inoculated with strains of *B. subtilis*. Some of the other species also gave this slightly putrid and astringent flavour after months of incubation, but the flavour development was not consistent.

(3) In addition, some strains of *B. subtilis* after many months' storage seemed to have thickened evaporated milk and to have produced a soft clot when compared with the condition of the control cans. It appeared that *B. circulans* had also thickened or formed small clots in evaporated milk. In interpreting this change in consistency allowance must, however, be made for the effect of the high temperature of incubation in causing normal thickening. In addition the heating of the outside of the can both at the time of inoculation and of examination may have been responsible for the observed differences in the consistency of the milk.

(4) All the cans inoculated with the four strains of *B. licheniformis* were very slightly "blown" after a period of from 2 weeks to 3 months. The consistency was, however, normal, although one or two cans had a slightly proteolytic flavour. It was thought at first that contamination had occurred but further investigation removed all possibility of this. *B. licheniformis*, although it does not usually produce gas from carbohydrate fermentation, can produce small gas bubbles between the glass and agar in dextrose agar stab cultures, and it is also the most tolerant to lack of oxygen of all the cultures examined. It seems probable, therefore, that *B. licheniformis* may produce a slight "swell" in these canned dairy products, especially as the plate counts which are given below were very high after 7 weeks, which indicated that growth had probably taken place in the tins.

Culture *B. licheniformis* strain	Inoculated into	Plate count per ml. after 7 weeks' incubation at 37° C.
No. 91	Canned cream	21,000
	Evaporated milk	43,000
No. 92	Canned cream	Approx. 60,000.
	Evaporated milk	32,000
No. 94	Evaporated milk	400

It will be seen that these samples with one exception had plate counts of over 20,000 per ml. In comparison plate counts of the bacteria causing bitterness and thinning (13) were very low. Usually after a period of from 5 to 6 weeks' storage at 37° C. the plate counts from such inoculated cans were under 1000. Plate counts made from any other cans of cream or evaporated milk which had been inoculated with other cultures in order to induce spoilage were also low.

(5) The aerobic sporeformers inoculated into cans of cream and evaporated milk survived for long periods even at the high storage temperature of 37° C.

During the first 6 months of incubation the bacteria previously inoculated into the cans were recovered from practically every tin examined. After 18 months, however, the recovery of bacteria, even from the large inocula used, was exceptional. It seems probable that if a larger number of transfers from the can contents into media had been made a higher number would have been shown to contain viable bacteria. This point is apparent from the results of an examination made of a can of roasted veal over 100 years old by Wilson & Shipp (14). They found that out of sixty-four tubes of media inoculated from the can contents all were sterile except five which contained strains of aerobic sporeformers.

The above results show that the aerobic sporeformers when inoculated into canned milk or cream may be responsible for a variety of defects, varying from very definite faults such as bitterness and thinning to less evident defects such as minor changes in flavour and consistency. The results also show that, while in some cans an organism may not cause spoilage although it apparently survives for long periods after inoculation, when inoculated into other cans the organism may affect the product. It is clear, therefore, that under commercial conditions the manufacturer should aim at a sterile pack, and it seems obvious from the survey undertaken that this should be ordinarily quite attainable.

The variable production of spoilage in canned cream and evaporated milk by the inoculation of these aerobic sporeformers and the variation in the extent of defects when these occur (as reported above), probably accounts for the considerable difference of opinion in the past regarding the connexion between aerobic sporeformers and deterioration in canned foods. Cameron & Esty (15) have reviewed this fully in their paper on the spoilage of non-acid canned foods. They examined the effect of inoculating canned milk with 6 different strains of aerobic sporeformers and reported that no perceptible difference was noted up to 200 days' storage at room temperature. On the other hand, Savage & Hunwicke (1) claimed that when these bacteria were added to experimental tins in large numbers they might be able to produce enzymes and promote clotting with considerable acid production. However, they stated that when present in small numbers and probably only as spores (as in some shop samples or in lightly inoculated experimental tins) they did not multiply but remained comparatively harmless. These authors concluded that aerobic sporeformers never caused gas production and very rarely unsoundness, but that it was possible in some circumstances (which they did not elucidate) "that the bacteria might be able to multiply and produce sufficient acid to cause a solid clot". Savage & Hunwicke, as well as Cameron & Esty, apparently expected acid development to be brought about by the growth of the aerobic sporeformers which they inoculated into canned milk. However, the aerobic sporeformers examined in the present study (with the single exception of *B. circulans*) failed to change the reaction of litmus milk during early incubation or occasionally turned it slightly more alkaline, while on ageing all the milk

cultures showed a definite increase in alkalinity. A clot was either produced before digestion commenced or during digestion, but it was never hard like an acid clot and could be easily broken up on shaking. It appears, therefore, that faults such as are induced by inoculating streptococci, coliform organisms or anaerobic sporeformers into sound cans are not to be expected from the action of the commonest species of aerobic sporeformers obtained from canned cream and evaporated milk. This conclusion is borne out by the results obtained with inoculated cans, as has already been noted.

(c) *Note on the relationship of the aerobic sporeformers to curdling in evaporated milk.*

Before leaving the general subject of the aerobic sporeformers and spoilage it seems desirable to consider in some detail certain reports regarding the occurrence of curdling due to these organisms. Two different types of curdling will be mentioned.

(i) *"Sweet" curdling or curdiness without increase of acidity.* Reports of this defect are of interest because many of the organisms already described were, so far as classification was possible, of similar species to those reported as causing sweet curdling, and therefore they were probably potential "sweet" curdlers.

Thayer(16) investigated non-acid coagulation of evaporated milk. He reported that *B. subtilis*, the hay bacillus, figured actively in this spoilage, and that the thermal death-point of the spores in milk was between 117 and 122° C. Hammer & Hussong(17) also identified *B. cereus* as responsible for curdy evaporated milk. They found that cultured evaporated milk in cans curdled slowly at 37° C. without change in odour and flavour unless more air was admitted, when curdling occurred rapidly with the production of a bad odour and flavour. A typical *B. cereus* was again isolated by Kelly(18) from evaporated milk. The defective milk had a custard-like clot on top but no other change. In addition, two strains of an organism resembling *B. vulgatus* which were obtained from two widely separated districts were reported by Morrison & Rettger(19) to cause spoilage in evaporated milk.

(ii) *Acid curdling or curdiness with increase of acidity.* The curdling of evaporated milk as a result of the high acidity produced by aerobic sporeformers in the cans has also been reported. Hammer(20) isolated a rod-shaped bacillus, *B. coagulans*, from this spoilage, and found that although it grew slowly at 37° C. it developed rapidly at 55° C. and that at 50° C.(21,22) it could clot milk in 3–4 days. Hussong & Hammer(23), investigating sour curdled evaporated milk in two separate condenseries, found the causative organism to be *B. calidolactis* which they had previously isolated from a plant drying skim milk. This sporeformer was of thermophilic character and failed to grow in milk at 37° C., but rapidly coagulated milk at 55° C. in under 20 hr.

In the present study, with the possible exception of two strains of *B. circulans* which produced an acid reaction with or without curdling in litmus

milk, no sporeformers were recovered from the coagulated defective samples which produced any appreciable acidity on reinoculation into milk media. Nevertheless, organisms producing acid curdling have been mentioned because sporeformers of this type have been obtained in fairly large numbers from spray-dried milk powder plates incubated at 55° C., and thus it would appear that in the bulk incoming milk at the factory they may be fairly common. The fact that none were, however, isolated even after incubating inoculated media at 55° C., a temperature at which both *B. calidolactis* and *B. coagulans* develop rapidly, would indicate that their survival in evaporated milk or canned cream under ordinary conditions in this country is rare and that as a cause of spoilage in these two products they are unimportant.

II. SWEETENED CONDENSED MILK

The manufacture of sweetened condensed milk does not involve complete sterilization, and this product invariably contains viable organisms. Under normal conditions the high sugar content, by exerting a high osmotic pressure, tends to plasmolyse the bacterial cells, which are effectively prevented from multiplication. Defects only occur when micro-organisms particularly resistant to the high sugar content survive the processing or gain access to the milk after it leaves the condenser.

A total of about eighty samples of condensed milk has been examined. Some samples were from general sources or from the special factories, but a few were received in connexion with complaints regarding the quality of the product.

Technique of examination

The opening of cans, culturing, and examination of the can contents and identification of bacteria were carried out as outlined for evaporated milk. In addition, the plate count and the presumptive coliform tests (24) were made on several of the samples so that the numbers of organisms present could be ascertained. It was difficult to pipette the milk, but this difficulty was reduced by warming the can in water prior to opening. A warm first dilution blank was also used to aid dilution.

Discussion

(1) *Incidence of defective cans*

Of the thirty-six general samples of full-cream and skim-sweetened condensed milk examined only three were defective. The number of samples tested was not sufficient to predict what percentage of defective samples might be expected in commercial practice. Two cans were "bloated" and one had a putrid flavour suggestive of bacterial proteolysis and was considerably thinned. Several other samples did not have the freshness of flavour which is usually characteristic of the product and could hardly be considered as first-class, though they could not be definitely classed as "defective". Plate counts (Table IV) made on some of these samples varied, the highest recorded (that of the putrid sample) being 267,000 per ml. of condensed milk. In addition, this sample gave a positive reaction to the presumptive coliform test in 1/10 ml.

amounts. As noted by other workers, many of the plate counts were very low, especially after the samples were several months old.

Table IV. *Sweetened condensed milk samples*

Plate count per ml.	Presumptive *B. coliform* test	Remarks regarding flavour and consistency of sample
3,360	+ve 1/10 ml.	Lacked freshness of flavour
8,400	−ve 1 ml.	Do.
25,600	−ve 1 ml.	Good
267,000	+ve 1/10 ml.	Putrid and thin
17,800	−ve 1 ml.	Good
50,600	−ve 1 ml.	Good
	12 samples about 18 months old when tested	
Less than 500	−ve 1 ml.	Several of these cans had "buttons" and were thickened

About forty tins were examined from one factory which had encountered "buttons". The examinations were made after several months' storage, when sufficient time had elapsed for the defect to develop. The extent of this outbreak was considerable, since 77% of the cans sampled at random from 15 different days' manufacture spread over several months showed "buttons".

(2) *Types of defective samples encountered*

(i) *Gassy fermentation.* In both the "bloats" examined sucrose-fermenting yeasts were responsible for the spoilage. Large numbers of bacteria of the staphylococci type were also isolated but did not produce gassy fermentation on reinoculation into the sound product. The presence of these bacteria was therefore without significance so far as gas formation was concerned. Further samples from the same sources were not available so that it was not possible to investigate the extent of spoilage in greater detail. It has been reported (see reviews in (1) and (2)) that when "bloats" occur in condensed milk the spoilage is normally caused by sucrose-fermenting yeasts.

(ii) *Progressive thickening.* Thickening was only found in cans which were opened in connexion with the outbreak of "buttons". It was not found possible to isolate any bacteria which were capable of reproducing thickening. It seems probable, as none of the samples were unduly acid, that the fault was either associated with the formation of "buttons" or more probably that it was of physico-chemical origin, since the latter type of defect is more apparent on ageing and the samples were from 9 to 18 months old when examined.

The thickening was obviously not of the same type as has been described by Rice & Downs(25) and by Downs(26). They showed that certain cocci, similar to *Staphylococcus albus*, could promote progressive thickening and that such bacterial thickening was associated with increased acidity. A sucrose in water ratio of 64·5 was necessary to prevent these cocci from growing. Downs considered that acidity alone was not responsible for thickening, but that the action of a rennin-like enzyme produced by the cocci was also instrumental.

(iii) "*Buttons*". This fault has been shown by Rogers *et al.*(27) to be due to the growth of a mould *Aspergillus repens* and by Knudsen(28) to be due to

Aspergillus glaucus and other moulds. The so-called "button" consists of a reddish brown cheesy curd mass $\frac{1}{4}$–$\frac{3}{4}$ in. in diameter. The defect develops on storage, and after 8 to 10 weeks "buttons" have assumed a definite form. The growth of the mould itself probably ceases after 2–3 weeks, since at later stages mould cannot be isolated from the "buttons" easily, though the "button" continues to develop from the activity of a clotting enzyme.

From the outbreak already mentioned the "button" formation was similar to that described above, except that occasionally the hard curd tended to spread in an indefinite shape round the edges of the can. Three moulds (M 1–3) which appeared to be species which have not previously been reported as causing "buttons" were isolated. In view of the difficulties of identifying many species of mould, and particularly members of the genus *Penicillium*, without specialized knowledge of the whole group, the author has considered it unwise to attempt to name the species. It is well known that many mould forms are considerably modified by the substrate in which growth occurs and that other conditions also are able to change the type of growth, and thus a single description or examination is often insufficient to enable identification, especially if the variations in form which may occur within the species are not appreciated.

Mr George Smith (29) has kindly investigated the characteristics of these cultures and he has reported as follows:

M 1 is a species of *Actinomyces* which is very similar in structure to some of the common soil forms. The hyphae and spores are, however, about $2\frac{1}{2}$ times the usual diameter. There seems to be no previous reference in the literature to such a strain of *Actinomyces*, and Smith has never before encountered a culture like it.

M 2 is a strain of *Penicillium terrestre* Jensen, and it is classed in Thorn's group Asymmetrica-Funiculosa.

M 3 is also a species of the *Penicillium* genus.

Each culture was inoculated separately into sweetened condensed milk and incubated at 22° C. to confirm the fact that it was the cause of "button" formation. The condensed milk was first filled into small, wide-neck glass bottles which were stoppered with cotton wool and heated in a bath to 140° F. for 30 min. in order to destroy any mould spores which might have gained entrance during the transfer. After this heating the bottles were cooled and when the moulds had been inoculated the bottles were stoppered with sterile rubber stoppers and sealed with paraffin wax to ensure that they were airtight. The volume of air space inside was similar to that which would occur in a commercial can. The development of mould was observed through the glass and was first evident with *M* 1 after 2 weeks' incubation. This mould formed yellow brown colonies in the initial stages of growth, whereas the other two cultures were green. The growth of M 3 was not visible until after 3 weeks' incubation at 22° C., while M 2 developed even more slowly.

As the moulds grew on the condensed milk and even after development

appeared to have been arrested, a reddish brown to orange discoloration increased under the original growth and spread around it. The same kind of discoloration occurred with all three cultures whether the mould had been green or brown originally. The bottles of cultured condensed milk showed considerable discoloration over the major part of the surface of the milk after 9 months' storage. Hard cheesy masses of curd were evident, especially round the edges of the bottles, and some samples were somewhat thick although the control samples remained unchanged.

This epidemic of defective samples was, therefore, very similar to those already reported in the literature. It appears, however, that the causative organisms were different, since one culture was a species of *Actinomyces* while the other two cultures were classified as species of the genus *Penicillium*.

SUMMARY

1. A survey of evaporated milk and canned cream samples from three condensed milk factories and from general sources has been undertaken to determine to what extent non-sterility occurred in commercial practice. In addition to the examination of sound samples many others have been tested which were obviously defective or which were associated with defective packs. 100% of the tins examined from general sources were sterile. The factory samples, especially those of canned cream, had a lower sterility percentage, although it appeared that this was associated with some special circumstance of operation and that a sterile pack should ordinarily be quite attainable.

2. An account is given of the methods used for sampling the can contents and for the recovery of bacteria from unsterile cans and their identification. The necessity for a large inoculum in such sampling is emphasized, since although the bacteria may survive for many months in the tins, they may be present in small numbers.

3. Data are presented regarding the types of defective samples encountered and the associated bacteriological flora. "Bloats" in evaporated milk and thin bitter and putrid cream were the two main causes of spoilage encountered.

4. By using the heat resistance of the organisms which were recovered from the cans as a basis of classification the defects have been divided into two groups, namely (1) a mixed general flora which does not withstand high temperatures, and (2) a restricted heat resistant flora which is of the sporeforming type.

5. The spoilage due to non-sporeformers has been shown to consist chiefly of "bloats" and/or curdling. A wide variety of bacteria was isolated from cans showing these defects, since such organisms usually gain access to the can during the cooling process through leaks caused by bad seaming or faulty sealing. Strains of the genus *Escherichia* were, however, the commonest producers of gassy spoilage.

6. Although eighty aerobic or facultative sporeformers have been isolated from sound and defective cans, only two cultures of anaerobic sporeformers

have been obtained, both of which were from sound cans. The anaerobic sporeformers which survive processing are not normally, therefore, of any great commercial importance. The aerobic sporeformers were all of the same general type, 69% being strains of *B. subtilis* and 15% strains of *B. licheniformis*. When the condition of the contents in the cans from which these aerobic sporeformers have been isolated was defective, the spoilage varied from a smooth soft clot to a thin consistency, the smell and flavour being putrid or bitter. The relationship between aerobic sporeformers and the defects was investigated by the inoculation of thirty-one strains of these organisms into sound evaporated milk and canned cream. Inoculated cans were incubated for up to 18 months but a clear connexion between the inoculated organism and the defect was not found to exist in all instances. One outstanding defect has, however, been induced, i.e. the development of a bitter flavour associated with thinness. With some strains of *B. subtilis* a slightly putrid flavour developed slowly, but this was variable, and with some of the other aerobic sporeformers a soft clot was formed in evaporated milk. Slightly "blown" cans resulted in all cans inoculated with *B. licheniformis*.

7. The variable production of spoilage in evaporated milk and canned cream by the inoculation of these aerobic sporeformers and the variation in the extent of defects when they occur has been suggested as the probable cause of the considerable difference of opinion in the past regarding the connexion between aerobic sporeformers and deterioration in canned foods. This has been discussed together with the conflicting literature on the subject.

8. Some sweetened condensed milk samples which invariably contain viable organisms have also been examined for defects due to micro-organisms. Two "bloats" and one sample with a putrid flavour were encountered. In addition, three strains of mould, which were isolated from an extensive outbreak of "buttons", and which have not been previously reported as the cause of this defect, have been investigated.

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224. THE DECOMPOSITION OF CITRIC ACID BY *BETACOCCUS CREMORIS*

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(With 4 Figures)

Introduction

The characteristic odour of a good butter culture (starter) is the result of the combined action of lactic acid bacteria (*Streptococcus cremoris,—lactis*) and betacocci (*Betacoccus cremoris, Streptococcus citrovorus* or *paracitrovorus*). In a pure culture of these betacocci in milk no aroma is observed, so that the associated growth of lactic acid streptococci seems to be necessary for its formation. According to van Beynum (1), aroma formation takes place during the growth phase in pure cultures of betacocci in milk, acidified with organic or inorganic acids. From this is concluded that the only task of the lactic streptococci in a starter is a souring which makes the milk suitable for the formation of the characteristic odour by the betacocci.

The aroma betacocci may produce in their fermentation process: acetic acid, CO_2 and the "C_4 compounds" diacetyl, acetylmethylcarbinol and butyleneglycol. They attack the citric acid of the milk, as Hammer showed (2), and can ferment much larger quantities of this substance (1).

There is a distinct difference between the behaviour of the aroma betacocci in neutral and in acid media. In neutral media there is no aroma formation, no production of C_4 compounds and a slow decomposition of citric acid. In acid media there is aroma formation (when the culture is exposed to the air), production of C_4 compounds and a rapid decomposition of citric acid. Acetic acid and CO_2 are produced in neutral and in acid medium.

The decomposition of citric acid at various acidities is shown in Fig. 1. (In Exp. VI of this series the decomposition begins so very late because of the prolonged lag period in the acid medium.)

According to Hammer and his co-workers (2) acetic acid originates from the citric acid. This was confirmed and in previous work at this station (1) it was found that from 1 mol. of citric acid, 2 mol. of CO_2 and about 1·5 mol. of acetic acid are formed.

Diacetyl, which gives starters their characteristic smell, is only found when the growing cultures are exposed to air. In an anaerobic souring process no odour and no production of diacetyl can be observed. Therefore, if a butter culture is soured aerobically without stirring, diacetyl can only be detected in the surface layer.

Acetylmethylcarbinol is formed in an acid medium aerobically as well as anaerobically. After being formed, diacetyl and the carbinol may be destroyed in the cultures. The rapidity with which the carbinol disappears depends on the acidity and on the strain used. It is slow at high acidities and the destruction can be prevented by adding an excess of acid to the culture after the formation of these substances.

According to Hammer *et al.*(3) the destruction of carbinol is due to the reduction of this substance to 2-3-butyleneglycol. We could confirm this.

The present investigation deals with the mechanism involved in the breakdown of citric acid to C_4 compounds, acetic acid and CO_2 in neutral and in acid media.

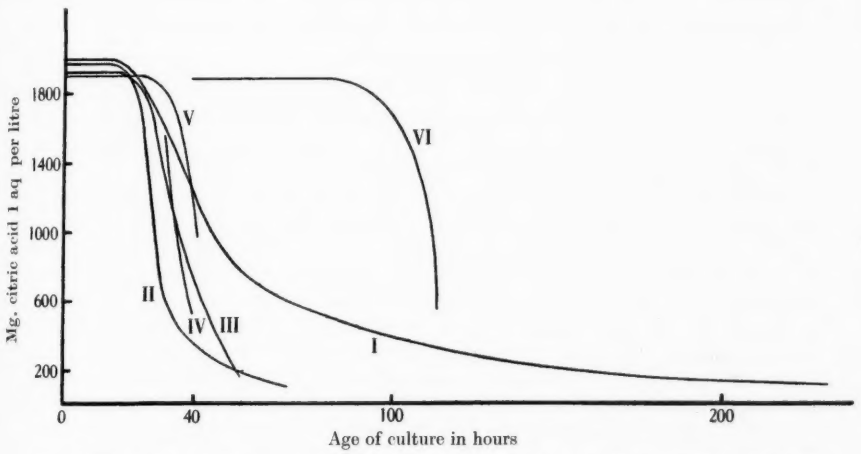


Fig. 1. Rate of citric acid decomposition in milk by *Bc. cremoris*. I, in skim milk; II, in skim milk with 1·67 ml. *N*/1 lactic acid per 100 ml.; III, in skim milk with 3·00 ml. *N*/1 lactic acid per 100 ml.; IV, in skim milk with 4·00 ml. *N*/1 lactic acid per 100 ml.; V, in skim milk with 5·00 ml. *N*/1 lactic acid per 100 ml.; VI, in skim milk with 6·33 ml. *N*/1 lactic acid per 100 ml.

Experimental technique

(1) *Estimation of acetic acid*(4)

50 ml. milk culture + 50 ml. water and 10 ml. *N* sulphuric acid are distilled with some drops of oleic acid to prevent foaming till 50 ml. distillate are obtained. Then 50 g. of hot water are added to the liquid in the distillation flask and another 50 ml. distilled off and so continued. The distillation portions are titrated with *N*/50 NaOH. The first distillate contains 32·7 % of the total amount of acetic acid, present in the 50 ml. culture, the second one 22·0 % and the third one 14·8 %. When the culture only contains acetic acid as a volatile acid the concentration of acetic acid calculated from the three distillates must be the same.

(2) *Estimation of carbon dioxide*

Dilute sulphuric acid is added to the culture and the CO_2 is driven out by bubbling CO_2-free air through it. The CO_2 is absorbed in U-tubes with soda-lime and weighed.

(3) *Diacetyl*

A quantity of at least 50 ml. milk culture, diluted with 50 ml. water, is distilled in a stream of carbon dioxide to prevent the oxidation of other substances to diacetyl, which may take place at the high distillation temperature. The distillate is collected in a tube containing 1 ml. 10% $NiCl_2.6$ aq.; 2 ml. 20% hydroxylamine hydrochloride; 2·5 ml. 20% sodium acetate ($3H_2O$) and 0·5 ml. $Na_2CO_3.10H_2O$. Diacetyl is present when red needles of Ni-dimethylglyoxime are formed.

(4) *Acetylmethylcarbinol + diacetyl*

From a mixture of 50 g. culture, 25 ml. water and 50 ml. 20% $FeCl_3$, 75 ml. are distilled off into a flask, containing the Ni-salt mixture mentioned above. The precipitate is filtered after 24 hr., washed and dried at 105° C. As we found that with this method only 80% of the carbinol is found as glyoxime, a correction for this loss should be made (factor 1·25).

A rough estimation of carbinol + diacetyl can also be made with Hammer's creatin test(5).

(5) *Estimation of butyleneglycol*

The method of Brockmann & Werkman(6) was used. A modification was necessary, however, because in milk, in which no butyleneglycol was present, a blank value of 270 mg./l. of this substance was found. This is due to a destruction of a part of the lactose when milk or milk serum is steam distilled with a large quantity of carbonate. In order to prevent this destruction of the lactose the butyleneglycol must be distilled off from a liquid, made only slightly alkaline. In our method 50 g. culture are neutralized with *N* NaOH and 1 ml. NaOH extra added. This is brought into a 500 ml. flask (rinsing with 10 ml. water) containing 30 g. NaCl. One litre is now distilled off with steam, while the volume is kept as constant as possible. In 500 ml. of the distillate the butyleneglycol is estimated as in (6). In the oxidation process with KIO_4 from 1 mol. of butyleneglycol, 2 mol. of acetaldehyde are formed, whereas from 1 mol. of carbinol 1 mol. of aldehyde is formed. The end-point of the titration with methyl-orange is estimated with the aid of a control of a *p*H of 4·2.

With this method the blank value in milk, containing no butylene-glycol, is only 20–30 mg./l.

(6) *Citric acid*

We made use of an improved Kunz method (7,1). To 50 g. of a culture were added 10 ml. sulphuric acid (1 : 2), 5 ml. of a solution of 106 g. $K_4FeCy_6.3H_2O$ per l. and 5 ml. of a solution of 238 g. Zn-acetate ($3H_2O$) + 30 g. glacial acetic acid per litre, and the whole made up to 200 ml. After filtering, 150 ml. of the filtrate were placed in an Erlenmeyer flask of 300 ml., fitted with a glass stopper, and 2 ml. 40% KBr-solution, 50 ml. H_2SO_4 (1 : 2) and 25 ml. saturated bromine water added. The citric acid was then oxidized by adding 10 ml. 5% $KMnO_4$ solution in drops with agitation. With high citric acid concentrations or when lactic acid is present more $KMnO_4$ is needed. After 5 min. the excess of bromine is removed with a ferrous sulphate solution. The penta-bromo-acetone is filtered into a weighed crucible the next day, washed with dilute H_2SO_4 and a small quantity of water and dried over P_2O_5 in the dark. A correction should be made for the solubility of the C_3OHBr_5 and for the volume of the casein precipitate. In skim milk the content of citric acid ($1H_2O$) is calculated as 12·25 $(p+q)$ mg./l. milk, where p is the quantity penta-bromo-acetone.

Citric acid as the source of the aroma

Betacoccus cremoris does not thrive well in synthetic media. However, in order to prove that citric acid is the constituent from which the aroma is

derived, a citric acid free medium was required. We used a special medium (PM) consisting of:

Water
1% peptone Poulenc
2% lactose
0·05% K_2HPO_4 and 0·02% $MgSO_4$
0·35% Na-acetate 3 aq. as a buffer
1·5% yeast autolysate
} *p*H 6·0

Table I shows that homofermentative lactic streptococci produce no appreciable amount of acetylmethylcarbinol from citric acid. The small quantity formed by some strains seems to originate from sugar. The betacocci do not produce carbinol because the medium in this experiment was neutral. Only *Str. citrophilus* (8) produces carbinol from the citric acid; but this streptococcus resembles a mixed culture of betacocci and streptococci.

Table I. *Cultivation of various bacteria in PM at* 21° C.

	4–5 days, cultivation in			
	PM		PM + 0·31% Na-citrate	
Culture	Carbinol	Growth	Carbinol	Growth
Str. cremoris IV	–	+	–	+
,, XVI	(+)	+	(+)	+
Str. lactis 15	–	+	–	+
,, 24	(±)	+	(±)	+
Str. citrophilus	–	+	+ + + +	+
Bc. cremoris a	–	+	–	+
,, *a* 1	–	+	–	+
,, *a* 5	–	+	–	+
,, *a* 7	–	+	–	+
,, *d*	–	+	–	+

In cultures of betacocci in acidified PM or in mixed cultures of betacocci and streptococci, in which a certain acidity is produced by the streptococci, the addition of citric acid gives rise to the formation of aroma and carbinol as is shown in Table II.

Table II. *Aroma and carbinol formation with citric acid*

	ml. *N*/1 lactic acid added per 100 ml.	PM + 0·31% Na-citrate		PM	
Culture	PM	Odour	Carbinol	Odour	Carbinol
Str. IV + *Bc. a* 1	—	Aroma	+ + +	No aroma	–
Str. XVI + *Bc. a* 1	—	Aroma	+ + +	No aroma	–
Str. 15 + *Bc. a* 1	—	Aroma	+ +	No aroma	–
Bc. a 1	0·2	No aroma	–	—	—
Bc. a 1	0·6	Aroma	+ +	—	—
Bc. a 1	0·8	No aroma	–	—	—

This proves that citric acid is the source of the aromatic odour and of the carbinol.

In milk cultures too, it was found that citric acid is necessary for the development of aroma. It is known that under anaerobic conditions the

cultures never produce an aromatic odour but citric acid is just as well decomposed. The anaerobic fermentations were carried out in glass-stoppered flasks, filled up to the top. After some time the milk culture was poured out into a dish in a thin layer so as to continue the process of fermentation aerobically.

One of our experiments is described in Table III. A mixed culture of *Str. cremoris* IV and *Bc. cremoris a* 1 was cultivated anaerobically in stoppered flasks. The milk was inoculated on 12 February 1935. The contents of each flask were poured into two dishes after different incubation times. To the liquid in one of the dishes was added 5 ml. 7% Na-citrate per 100 ml. culture and to the liquid in the other dish nothing was added. From time to time the cultures in the dishes were tested for aroma as shown in Table III.

Table III. *Milk inoculated on* 12 *February* 1935 *with* 2% *of a starter, consisting of* Str. cremoris IV *and* Bc. cremoris *a* 1. *Cultivated anaerobically*

Poured out into dishes on	13. ii at 11.30 a.m.		13. i at 11 p.m.		14. ii at 10 a.m.	
Citric acid present in mg./l.	632		0		0	
Added to the liquid in the dish	—	Citrate	—	Citrate	—	Citrate
Odour on 13. ii at 3 p.m.	Aroma	Aroma	—	—	—	—
,, 14. ii at 11 a.m.	Aroma	Aroma	None	Aroma	None	None
,, 15. ii at 11 a.m.	Aroma	Aroma	None	Aroma	None	Aroma

This table shows that aroma is only formed when citric acid is still present in the original culture or when citric acid is added to a culture in which all the original citric acid has been fermented.

Reaction products of the citric acid fermentation

The relation between the citric acid fermented and the amount of fermentation products was studied in experiments with the medium PM to which citric acid was added. A number of experiments was made with *Streptococcus citrophilus* as this bacterium thrives better in the synthetic medium than the betacocci.

Table IV shows that there is a strict proportionality between the citric acid fermented and the acetic acid produced. Such a relation with the carbinol content was not found because a part of this compound was reduced and butylene glycol was not determined in this experiment.

Table IV. Str. citrophilus *cultivated in PM with added Na-citrate*

g. Na-citrate per litre fermented	Acetic acid formed out of citrate		mg./l. carbinol	
	mg./l.	Millimol from 1 mM. citric acid	5 days	10 days
1·0	175	1·02	Traces	Traces
1·5	296	1·15	208	39
2·0	374	1·10	257	95
2·5	495	1·16	293	131
3·0	580	1·13	—	184

The data of an experiment with a betacoccus are given in Table V.

Table V. *Amount of acetic acid produced from citric acid by* Bc. cremoris *a* 1

g./l. Na-citrate fermented	In PM		In PM + lactic acid	
	mg./l.	Millimol per 1 mM. citric acid	mg./l.	Millimol per 1 mM. citric acid
1·0	283	1·65	276	1·62
1·5	400	1·56	422	1·65
2·0	561	1·64	500	1·47
2·5	—	—	655	1·54
3·0	760	1·49	818	1·60

In this table no data concerning acetylmethylcarbinol are given because this was not produced.

From Tables IV and V we conclude that there must exist a reciprocal relation between carbinol and acetic acid, for when carbinol has been produced the acetic acid content appears to be lower than when no carbinol production took place.

There are, however, two objections to this conclusion, viz.

(1) In these experiments two different kinds of bacteria were used.

(2) In one of the experiments no carbinol had been formed, so it is to be doubted whether it is allowed to extend the rule of the reciprocal relation to experiments in which there are only quantitative differences in the carbinol content.

Tables VI and VII show the relationship between acetylmethylcarbinol and acetic acid in experiments with *Str. citrophilus*. Similarly, Tables VIII and IX demonstrate the same aspect for *Bc. cremoris*.

Table VI. Str. citrophilus *in PM with Na-citrate. Determination after* 5 *days' cultivation*

g./l. citrate fermented	Formed from the citrate	
	mg./l. acetic acid	mg./l. carbinol
1·5	378	46
3·0	554	224
3·0	594	249

From the data of Table VI the millimols acetic acid and carbinol have been calculated (Table VII). In this calculation the carbinol figures have been multiplied by 1·5, because we observed that *Str. citrophilus* reduced one third of the carbinol in the course of 5 days. The molecular weight of Na-citrate was taken as 352 as the water content was slightly over $5H_2O$.

Table VII. *Millimols of fermented substance and fermentation products from Table VI*

Citrate fermented	Acetic acid	Carbinol	Acetic acid per millimol	Carbinol citrate
4·25	6·3	0·79	1·48	0·19
8·5	9·2	3·81	1·09	0·45
8·5	9·9	4·24	1·17	0·50

Table VIII. Str. lactis 15 + Bc. cremoris *a* 1, *cultivated in lactose broth*

Exp.	g./l. citrate fermented	Produced from the citrate		
		mg./l. CO_2	mg./l. acetic acid	mg./l. carbinol
a	0·74	166	171	34
b	1·47	331	313	110
c	2·20	517	436	185
d	1·47	329	290	113
e	1·47	343	316	103
f	2·92	693	554	233

The amounts of the fermentation products have been calculated by the difference between two experiments, one with and the other without citrate.

The millimols of the products in the experiment of Table VIII are tabulated in Table IX. The carbinol data have not been multiplied by 1·5 as *Bc. cremoris* *a* 1 does not reduce carbinol.

Table IX. *Number of molecules of fermentation products from one molecule of citrate*

Exp.	Acetic acid	Carbinol	Carbon dioxide
a	1·35	0·184	1·79
b	1·24	0·299	1·80
c	1·16	0·336	1·88
c–b	0·995	0·411	2·05
d	1·15	0·307	1·78
e	1·26	0·280	1·86
f	1·12	0·319	1·90
f–e	0·97	0·360	1·94

As may be seen from Table IX, the amount of carbon dioxide is fairly constant and nearly equivalent to 2 mol. from 1 mol. of citrate. Tables VII and IX prove the conclusion, mentioned above, that the amount of acetic acid is high with low carbinol content and vice versa. This relation between acetic acid and carbinol in the experiment of Table IX is illustrated in Fig. 2.

When the amounts of carbon present in the fermented citrate and in the fermentation products are calculated, we see from Table X that nearly all the carbon is recovered in the fermentation products acetic acid, acetylmethylcarbinol and carbon dioxide. The slight loss of carbon will be discussed later.

Table X. *Carbon balance of the data of Tables VIII and IX*

Exp.	Found in the products mg. carbon per litre				Citrate fermented
	Carbon dioxide	Acetic acid	Carbinol	Sum	
a	45·3	68·4	18·5	132·2	151·4
b	90·3	125·2	60·0	275·5	300·7
c	141·0	174·4	101·0	416·4	450·0
c–b	50·7	49·2	40·9	140·8	149·3
d	89·7	116·0	61·6	267·3	300·7
e	93·5	126·4	56·2	276·1	300·7
f	189·0	221·6	127·1	537·7	597·3
f–e	95·5	95·2	70·9	261·6	299·4

From these and other experiments we can conclude that from one molecule of citric acid are produced from 1 to 1·5 mol. of acetic acid and from 0·5 to 0 mol. of carbinol. The reciprocal relation between acetic acid and carbinol suggests that carbinol and a part of the acetic acid are formed from the same intermediate substance.

As the carbinol is only produced in acid media the reaction in neutral media represents the extreme case of the reciprocal relation between acetic acid and acetylmethylcarbinol. Then the amount of carbinol is 0 and the amount of acetic acid is maximal.

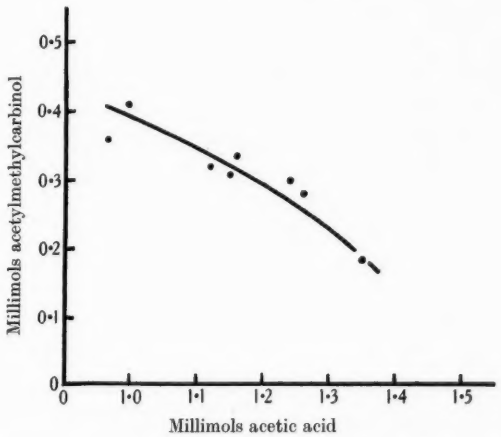


Fig. 2. Relation between acetylmethylcarbinol and acetic acid in mixed cultures of *Str. lactis* 15 and *Bc. cremoris a* 1 in sugar broth + citrate.

This can also be shown in milk cultures. In an early experiment of 1925 the end concentration of acetic acid in a pure culture of an aroma betacoccus in skim milk, acidified with 10, 20, 40 and 60 ml. *N*/1 sulphuric acid per litre was 1050, 1038, 903 and 833 mg./l. respectively. This confirms the above conclusions, as in the milk with 10 and 20 ml. sulphuric acid no carbinol could have been formed, whereas in the milk with 60 ml. sulphuric acid the carbinol concentration was highest.

The reciprocal relation between acetic acid and carbinol is also illustrated by experiments in which the aroma betacocci were inoculated (*a*) earlier than the lactic streptococci and (*b*) simultaneously (Table XI).

Table XI. Bc. cremoris *and* Str. cremoris *cultivated in milk.* 21° C.

Betacoccus inoculated	*Streptococcus* inoculated	mg. per litre		
		Citric Acid	Carbinol	Acetic acid
17. x. 36	19. x. 36	0	0	1190
18. x. 36	19. x. 36	0	0	1142
19. x. 36	19. x. 36	0	244	924

In the first and second experiments all or most of the citric acid had been fermented in the neutral medium, consequently without formation of carbinol. In these experiments the amount of acetic acid is higher than in the third experiment in which carbinol had been formed during the citric acid fermentation which took place in the acid medium.

The following conclusions may be drawn from these experiments:

(1) Carbonic acid, acetic acid and acetylmethylcarbinol are products of the fermentation of citric acid.

(2) The decomposition of the citric acid molecule gives rise to the formation of two molecules of carbonic acid; from 1·5 to 1 mol. of acetic acid and from 0 to 0·5 mol. of carbinol, depending on the acidity.

(3) The amounts of acetic acid and carbinol are interrelated. A large quantity of carbinol corresponds with a low acetic acid content and vice versa.

(4) Fermentation in a neutral medium is the extreme case of this reciprocal relation as here the maximal amount of acetic acid but no carbinol is formed.

(5) Carbinol and a part of the acetic acid originate from the same intermediate substance.

The intermediate substances of the citric acid fermentation

Probably the first step in the breakdown of the citric acid molecule is the splitting into 1 mol. of carbon dioxide, 1 mol. of acetic acid and 1 mol. of pyruvic acid:

$$COOH.CH_2.C(OH.COOH).CH_2.COOH \rightarrow CO_2 + CH_3.COOH + CH_3.CO.COOH.$$

This scheme was supported by experiments in which a milk culture of a good starter, after having been cultivated anaerobically till all citric acid had disappeared, was poured out into flat dishes and pyruvic acid added (Table XII).

Table XII. *Two starter cultures, having soured anaerobically, were poured out on* 17 *April* 1935 *at* 5 *p.m. Odour estimated* 18 *April*

Addition	Starter 1	Starter 2
Nothing	No aroma	No aroma
0·35% Na-citrate	Aroma	Aroma
0·056% Ca-pyruvate	No aroma	Aroma
0·112% Ca-pyruvate	Weak aroma	Aroma
0·224% Ca-pyruvate	No aroma	Weak aroma
0·058% Na-pyruvate	Weak aroma	Aroma
0·115% Na-pyruvate	Aroma	Aroma

The probability of pyruvic acid as an intermediate substance becomes still greater if it can be shown that with pyruvic acid these bacteria produce the same fermentation products as from citric acid.

Table XIII gives the data of an experiment with *Str. citrophilus* cultivated in PM with and without pyruvic acid (3038 mg./l. in the form of Na-pyruvate).

Table XIII. *Formation of acetylmethylcarbinol and acetic acid from pyruvic acid by* Str. citrophilus

mg./l.	PM	PM + pyruvate
Acetic acid	0	246
Carbinol	0	1110

From this experiment it may be calculated that 1 mol. of pyruvic acid has been split into at least 0·37 mol. of carbinol and at most 0·123 mol. of acetic acid. These figures are within the limits mentioned before, because, as the amounts of acetic acid and carbinol vary between 1–1·5 and 0·5–0 in the citric acid fermentation, these figures must be 0–0·5 and 0·5–0 with pyruvic acid.

Carbinol was also obtained when aroma betacocci were cultivated in PM + pyruvate acidified with lactic acid. With a mixed culture of streptococci and betacocci, pyruvic acid was split and carbinol formed. However, as there are lactic acid streptococci which attack pyruvate in this way, care must be taken that only those streptococci which do not attack pyruvic acid are used.

During the decomposition of the pyruvic acid carbon dioxide is also formed as could be shown with cultures of *Str. citrophilus* and with mixed cultures of streptococci and betacocci in PM + pyruvate. In this step the second molecule of carbon dioxide which is formed in the decomposition of the citric acid molecule is split off.

The pyruvic acid molecule breaks down as follows:

$$CH_3.CO.COOH \rightarrow CH_3.CHO + CO_2.$$

The reciprocal relation between acetic acid and carbinol can now be explained when we assume that the acetaldehyde reacts in two ways, viz.

(1) Condensation to acetylmethylcarbinol:

$$2CH_3.CHO \rightarrow CH_3.CO.CHOH.CH_3.$$

(2) Transformation to acetic acid and ethyl alcohol (Cannizaro reaction):

$$2CH_3.CHO + H_2O \rightarrow CH_3.COOH + CH_3.CH_2OH.$$

In neutral media only the second reaction takes place and from the citric acid molecule are formed 1·5 mol. of acetic acid, two molecules of carbon dioxide and 0·5 mol. of ethyl alcohol. (Ethyl alcohol could be detected in about the expected amount in neutral milk culture of aroma betacocci.)

The first aldehyde reaction can only occur in an acid medium, but in an acid medium a part of the aldehyde also reacts according to the second reaction.

The decomposition of the citric acid may be expressed by the formula:

$$C_6H_8O_7 + xH_2O \rightarrow 2CO_2 + (1+x)\,CH_3.COOH + (0{\cdot}5-x)\,CH_3.CO.CHOH.CH_3 + xCH_3.CH_2OH,$$

in which x may vary from 0 to 0·5; x is 0·5 in neutral media.

In this formula the acetylmethylcarbinol appears as one of the end-products of the fermentation. However, we know that carbinol may be reduced to butylene glycol, and consequently the determination of the carbinol content does not reveal how much carbinol really has been formed. The real amount of carbinol, produced during the fermentation, can only be found by adding to the carbinol, present in the liquid, the reduced carbinol, i.e. the butylene glycol. This correction was made in the calculations of Tables VI and VII, as we knew that during the experiment one third of the carbinol had been reduced. No correction was applied to the experiments of Tables VIII and IX as a *Bc. cremoris* strain was used which did not reduce carbinol at the high acidity of the medium concerned. Consequently the reciprocal relation exists between acetic acid and the C_4 compounds so that in the above formula for carbinol may be substituted "C_4 compounds":

$$C_6H_8O_7 + xH_2O \rightarrow 2CO_2 + (1+x)\,CH_3.COOH + (0{\cdot}5-x)\,C_4 \text{ compounds} + xCH_3.CH_2OH.$$

The value of x depends on the acidity of the medium as is shown in Table XIV. The higher the acidity the more the acetaldehyde reacts according to reaction 1 and less according to reaction 2.

Table XIV. *Production of* C_4 *compounds by the* aroma betacoccus *a* 1 *in skim milk, acidified with lactic acid*

ml. $N/1$ lactic acid per litre	mg. per litre		Millimols C_4 compounds
	Diacetyl + carbinol	Butylene glycol	
30	0	106	1·18
40	46	114	1·79
50	88	129	2·43
60	232	48	3·17

Fig. 3 shows a graph of this experiment. In this graph the results obtained with two other strains of aroma betacocci 19·1 and *d* have also been given. The value of x also depends on the strain used. The strain *d* is an exception amongst our collection of betacocci strains as it forms traces of butylene glycol in a neutral medium.

Thus it is seen that with higher acidities more C_4 compounds are produced. In a mixed culture of lactic acid streptococci and aroma betacocci, in which the betacocci attack the citric acid as the acidity increases the proportion C_4 compounds, acetic acid must be lowest in the beginning and increase during the souring process. This can be proved from the experiment of Table IX. In this table the figures for *c–b* and *f–e* represent the results of the decomposition of the amount of citric acid, fermented during the last period of the process, i.e. at the highest acidities.

If our assumption that the aldehyde reacts in two ways is true, in the same nutritive medium and with the same strain of aroma betacoccus the relation between acetic acid and C_4 compounds should be represented graphically by a

straight line when the results of experiments in milks of different acidities are compared.

Table XV contains the data of such an experiment. The first analysis of the fermentation products was undertaken as soon as possible after the disappearance of the citric acid. These results are represented by the line *A* of fig. 4. The second analysis was made one week later and its results are shown in line *B*. This line is perfectly straight. Here we meet with the difficulty that it

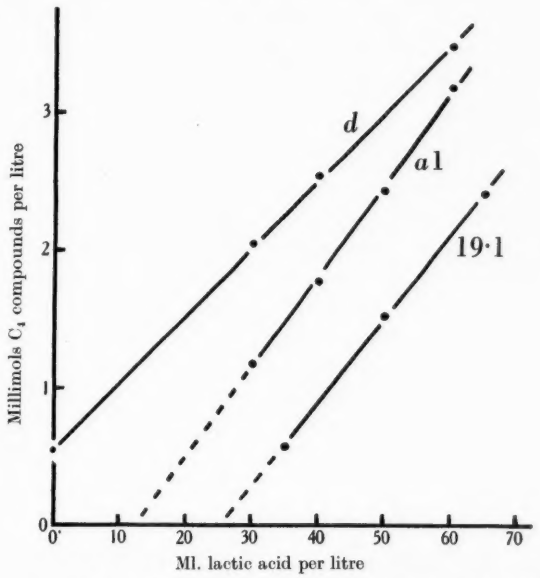


Fig. 3. Production of C_4 compounds by three strains of *Bc. cremoris* in skim milk, acidified with lactic acid.

is uncertain what part of the total amount of acetic acid is due to a decomposition of sugar. From the data of Table XV we conclude that the acetic acid production from sugar reaches its endpoint earlier in acid than in neutral

Table XV. Aroma betacoccus 19·1 *cultivated in milk acidified with lactic acid*

ml. *N*/1 lactic acid per litre	Analysed after days	Citric acid	mg./l. Acetic acid	mg./l. Diacetyl + carbinol	mg./l. Butylene glycol	Millimol C_4 compounds per litre
65	5	0	816	137	73	2·37
50	3	0	873	43	84	1·42
35	3	0	882	0	43	0·48
10	9	0	890	0	0	0
65	12	0	818	131	81	2·39
50	10	0	873	43	93	1·52
35	10	0	929	0	52	0·58
10	16	0	976	0	0	0

media. The uncertainty about the amount of acetic acid produced from citric acid is still greater in mixed cultures of aroma betacocci and lactic streptococci in which the streptococci also form a little acetic acid from sugar. In Fig. 4, line *C* represents the data of an experiment in which four other strains of aroma betacocci were inoculated into milk each with the same *Streptococcus*. From the acetic acid figures the amount of acetic acid produced by the *Streptococcus* alone in the same milk has been subtracted.

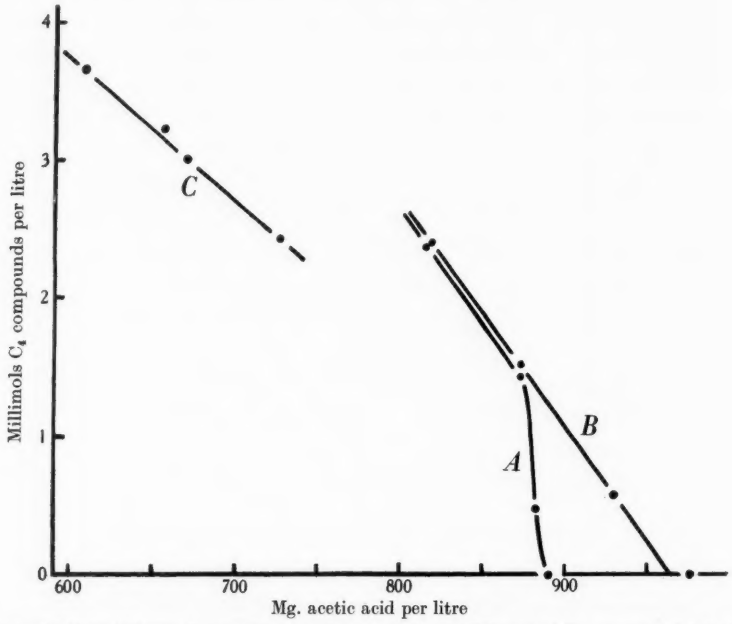


Fig. 4. Relation between C_4 compounds and acetic acid. *A*. Cultures of *Bc. cremoris* 19·1 in skim milk of different acidities at the moments that citric acid had just been totally fermented. *B*. The same a week later. *C*. Four strains of *Bc. cremoris*, each combined with the same strain of *Str. lactis*, cultivated in skim milk.

Formation of diacetyl

In the experiments mentioned above the carbinol figures of the tables always represent the sum of carbinol + diacetyl. As diacetyl is the principal aroma constituent it is therefore of great interest to know how it is formed. We know that oxygen is necessary for the production of diacetyl in the cultures and it is generally assumed that diacetyl is an oxidation product of acetylmethylcarbinol. In our opinion carbinol is not oxidized in the cultures:

(*a*) When synthetic carbinol was added to cultures of aroma betacocci in acidified PM or to mixed cultures of streptococci and betacocci in PM the smell of diacetyl was never observed.

(*b*) In mixed cultures of streptococci and betacocci in milk in which all citric acid has been fermented anaerobically without formation of diacetyl this

compound is not formed when the cultivation is carried on aerobically. When citric acid is not added no aroma formation takes place although carbinol produced anaerobically from the citric acid fermentation is present. The results of such an experiment are described in Table III. This experiment was undertaken to show that citric acid is the source of diacetyl but it also proves that diacetyl is formed neither by a chemical nor by a biological oxidation of carbinol, which was present in large quantities when the cultures were poured out into the dishes. Consequently it must be formed by the oxidation of an intermediate substance, probably acetaldehyde

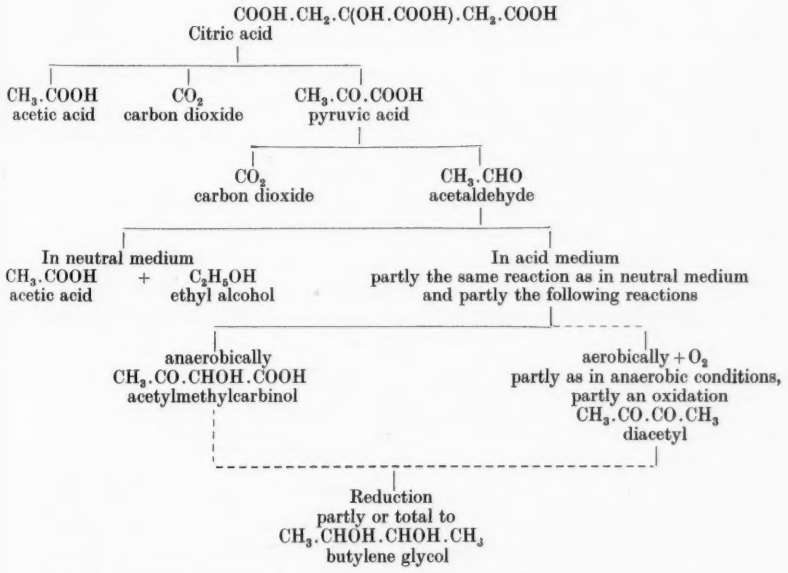
$$2CH_3.CHO + \tfrac{1}{2}O_2 \rightarrow CH_3.CO.CO.CH_3 + H_2O.$$

This oxidation must be regarded as a biological one.

The fermentation of citric acid

The decomposition of the citric acid molecule can be represented in the following scheme which includes the fermentation process of *Betacoccus cremoris* as well as that of *Streptococcus citrophilus*.

Scheme of citric acid fermentation



The whole process can be represented by the following equation:

$$1C_6H_8O_7 + (x-y)\,H_2O + \tfrac{1}{2}yO_2 + 2zH \rightarrow 2CO_2 + (1+x)\,CH_3.COOH + (0{\cdot}5-x-y-z)\text{ acetylmethylcarbinol} + y\text{ diacetyl} + z\text{ butyleneglycol} + x\text{ ethyl alcohol},$$

in which x may vary from 0 to 0·5 and the maximal value of z is $0{\cdot}5-x$. (In this case of maximal reduction $y=0$.) Generally y is only small.

In neutral media $x = 0{\cdot}5$, $y = 0$ and $z = 0$, and in acid media z diminishes with increasing acidity. z is small with non-reducing betacocci, but is large with strongly reducing strains.

The term $\frac{1}{2}yO_2$ on the left hand side of the formula means that the diacetyl is formed with the aid of atmospheric oxygen. The term $2z$H means that the reduction of diacetyl and carbinol to butylene glycol is only possible with the aid of a hydrogen donator outside the citric acid dissimilation process, e.g. some intermediate compound of the sugar decomposition.

The above scheme may be verified by comparing the amounts of carbon in the fermentation products. In the experiment of Table XV the decomposition of the citric acid took place in milk cultures at four different acidities. The carbon contents of the fermentation products have been calculated in Table XVI.

Table XVI. *Amount of carbon in the fermentation products of the experiment of Table XV*

	Acetic acid		Carbinol + diacetyl		Butylene glycol		Total amount of C per litre
Acidity	mg.	mg. C	mg.	mg. C	mg.	mg. C	
65	818	327·2	131	71·5	81	43·2	441·9
50	873	349·2	43	23·5	93	49·6	422·3
35	929	371·6	0	0	52	27·7	399·3
10	976	390·4	0	0	0	0	390·4

Carbon dioxide was not taken into consideration as it was not determined but it is known that its amount is independent of the acidity. We see from this table that the total amount of carbon in the fermentation products is not the same at the different acidities as might be expected. In Table XVI, however, ethyl alcohol was not mentioned, because it was not estimated, but its amount is dependent on the acidity. A correction for the amount of alcohol carbon can be calculated, for when in two experiments there is a difference in the number of millimols of acetic acid, the difference in the number of millimols of ethyl alcohol must be the same. This calculation has been made in Table XVII.

Table XVII. *Correction of Table XVI with regard to ethyl alcohol carbon*

	Acetic acid		Ethyl alcohol calculated		
Acidity	mg.	Difference	Difference in mg.	mg. C	Total amount of C per litre
65	818	—	—	—	441·9
50	873	55	42·2	22·0	444·3
35	929	111	85·1	44·4	443·7
10	976	158	121·1	63·2	453·6

The figures for the ethyl alcohol content in Table XVII consequently do not represent the real contents of alcohol but only represent the differences from the unknown alcohol content at acidity "65".

With this correction for ethyl alcohol the total amount of carbon in the fermentation products is found to be fairly constant as we see from Table XVII.

The loss of carbon in the carbon balance of Table X must be explained, too, by the production of some alcohol.

This scheme of the citric acid fermentation by *Betacoccus cremoris* explains qualitatively and quantitatively all phenomena so far observed in butter starters or other cultures in which this organism is active.

SUMMARY

The fermentation products of *Betacoccus cremoris* in neutral milk are acetic acid and carbonic acid. In acidified milk or in mixed cultures of this bacterium and lactic acid streptococci the products are acetic acid, CO_2, diacetyl, acetylmethyl carbinol and 2–3 butylene glycol. The latter three substances may be called "C_4 compounds". Diacetyl is only formed when an oxidation with atmospheric oxygen can take place. Carbinol is found in aerobic and in anaerobic cultures. It may be reduced to butylene glycol. This reduction is more complete at lower acidity, but also depends on the strain of *Betacoccus* used.

All these substances are formed from the citric acid of the milk. However, small amounts of acetic acid may be produced from sugar.

From 1 mol. of citric acid are formed: 2 mol. of carbon dioxide, 1–1·5 mol. of acetic acid, 0·5–0 mol. of C_4 compounds.

A reciprocal relation exists between the quantities of acetic acid and C_4 compounds. When the amount of acetic acid is high the C_4 compounds content is low. The higher the acidity of the medium in which the betacocci are cultivated the higher is the amount of C_4 compounds. This means that the fermentation in neutral media is the extreme case of the fermentation process. Then the production of C_4 compounds is minimal (0) and that of acetic acid is maximal (1·5).

It is highly probable that pyruvic acid is an intermediate product. With pyruvic acid, added to milk freed from citric acid by anaerobic fermentation, the formation of CO_2 and acetic acid, C_4 compounds and aromatic odour could be demonstrated.

Probably citric acid is decomposed as follows:

$$C_6H_8O_7 \rightarrow CO_2 + CH_3.COOH + CH_3.CO.COOH$$

and

$$CH_3.CO.COOH \rightarrow CO_2 + CH_3.CHO.$$

The aldehyde reacts in two ways:

(1) Condensation to acetylmethylcarbinol:

$$2CH_3.CHO \rightarrow CH_3.CO.CHOH.CH_3.$$

(2) Transformation to acetic acid and ethyl alcohol (Cannizaro reaction):

$$2CH_3.CHO + H_2O \rightarrow CH_3.COOH + CH_3.CH_2OH.$$

These two reactions explain the reciprocal relation between the quantities of acetic acid and C_4 compounds. In neutral medium only the second reaction occurs, in acid medium both these reactions take place, but at lower acidities less of the aldehyde reacts according to the first reaction than at high acidities.

In cultures of *Bc. cremoris* diacetyl is not formed as an oxidation product of acetylmethylcarbinol. It must be formed when oxygen interacts during the aldehyde condensation reaction:

$$2CH_3.CHO + \tfrac{1}{2}O_2 \rightarrow CH_3.CO.CO.CH_3 + H_2O.$$

Diacetyl and carbinol may be reduced in the cultures with the aid of a hydrogen donator, probably an intermediate compound of the sugar dissimilation.

In a series of experiments carried out at different acidities the amount of carbon in the various fermentation products was constant, independent of the amounts of each product.

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225. THE CASEIN NUMBER FOR DIAGNOSIS OF MASTITIS

II. THE EFFECT OF ADVANCED LACTATION, AND OF STORAGE AND PRESERVATION OF THE MILK SAMPLE

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In an earlier paper (1) it was shown that the percentage of casein nitrogen in the total nitrogen of the milk from individual quarters provides a valuable indirect method for the diagnosis of mastitis. This percentage was called the "casein number", and a figure of 78·0 or less was taken as indicating an infected quarter. The effect on the casein number of advanced lactation, the temperature of storage of the milk sample, and the use of preservatives has since been investigated and is dealt with in this paper.

The samples of milk were collected, and the determination of the total nitrogen and casein nitrogen, and the cultural examination for *Str. agalactiae*, carried out as described previously (1).

I. The effect of advanced lactation

The milk from healthy cows approaching the end of lactation undergoes many of the changes in composition found in mastitis-infected milk, and it was necessary to ascertain whether or not the casein number of milk from uninfected cows in advanced lactation is normal.

Samples of milk were taken from the twelve quarters of three cows A, B, and C, which were in their 11th, 10th, and 10th month of lactation respectively. The cows became dry 6, 7, and 1½ weeks after the sampling. The results are recorded in Table I, where they are compared with the results obtained for the same quarters when the cows were in full milk.

Table I. *Effect of period of lactation on the casein number*

Cow and quarter*	Cow in full milk: Bacteriological result	Total nitrogen (mg. per 100 ml.)	Casein nitrogen (mg. per 100 ml.)	Casein no.	Cow near end of lactation: Bacteriological result	Total nitrogen (mg. per 100 ml.)	Casein nitrogen (mg. per 100 ml.)	Casein no.
A. RF	–	542·5	422·7	78·0	–	736·9	514·5	69·8
RH	–	556·8	441·1	79·2	–	741·0	462·3	62·4
LF	+	544·7	416·2	76·4	+	753·3	517·2	68·7
LH	–	556·6	444·9	80·0	–	795·8	595·2	74·8
B. RF	–	661·5	529·0	80·0	–	731·5	567·4	77·6
RH	+	638·1	468·5	73·5	+	594·0	264·3	44·5
LF	–	658·0	524·3	79·8	–	735·9	560·7	76·2
LH	–	670·0	534·4	79·7	–	723·5	558·3	77·2
C. RF	–	528·6	415·4	78·6	–	786·8	513·8	65·3
RH	–	533·5	417·5	78·3	–	862·0	535·4	62·1
LF	+	547·4	382·2	69·9	–	768·7	580·4	75·6
LH	+	519·2	402·1	77·4	+	926·0	573·8	62·0

* RF, RH, LF, and LH = right fore, right hind, left fore, and left hind.

It will be seen that when the cow is nearing the end of lactation it is no longer possible to discriminate, by means of the casein number, between infected and uninfected quarters. The casein number of the uninfected quarters was below 78·0, and this, for cows in full milk, would denote infection. Hence the casein number method of diagnosis is not applicable to animals in advanced lactation, as it would give positive results in all cases.

The casein nitrogen and total nitrogen contents of eleven of the twelve milk samples increased towards the end of lactation, the mean casein nitrogen and total nitrogen changing from 448·2 and 574·4 to 543·5 and 778·3 mg. per 100 ml. respectively. The total nitrogen therefore increased by 36%, and the casein nitrogen by 21%. The soluble protein plus non-protein nitrogen increased from 126·2 to 234·8 mg. per 100 ml., i.e. by 86%, and this accounts for the abnormally low casein number of the milk of uninfected quarters near the end of lactation.

The right hind quarter of cow B was exceptional in that the total nitrogen and casein nitrogen contents decreased, but this was an infected quarter, the ability of which to elaborate casein had become seriously impaired, the casein number falling to 44·5. The left fore quarter of cow C is also exceptional; it is the only quarter to show an increase in casein number (69·9–75·6), and although infected when the cow was in full milk, it was found to be negative on the two occasions, near the end of lactation, when it was examined bacteriologically.

II. The effect of storage of the milk sample, and of the use of preservatives

In the original investigation on the casein number(1) the precipitation of casein from most of the samples was carried out within a few hours of milking, and the remainder were stored overnight at a low temperature. Occasions may, however, arise when it is not possible to analyse samples so soon after they have been drawn, and therefore an investigation was made of the effect on the casein number of storage (1) at room temperature, (2) in the refrigerator, and (3) in the presence of preservatives at room temperature.

Effect of storage at room temperature

The change in casein number on keeping milk at a room temperature of 16–18° C. is illustrated in Table II. There is a steady decrease in casein number, and this is appreciable before much acidity has developed, and long before the milk sample shows signs of thickening or curdling. It may be noted that when the milk sample had developed an acidity greater than 0·25% lactic acid by titration, the portion removed for precipitation of casein was adjusted to the *p*H of normal fresh milk, by the addition of *N*/10 NaOH, before the actual precipitation was made with acetic acid and sodium acetate. This ensured the correct final *p*H for the precipitation of casein.

Table II. *Effect on the casein number of keeping milk at a temperature of* 16–18° C.

Sample no.*	Casein number: Age of milk sample in hours				
	0	24	48	96	120
1	79·6	78·9	76·4	51·1†	42·1
2	79·9	79·7	79·2	78·1	—
3	78·9	77·2	76·2†	74·8	—
4	79·0	78·4	77·4	66·9	—
5	77·4	77·0	76·4	75·7†	—
6	74·2	72·4	70·0	48·8†	36·5
7	66·2	62·4	—	—	—
8	73·4	71·1	62·1	—	—

* Samples 1–4 uninfected, and 5–8 infected. † Milk samples coagulated at this stage.

For the detection of mastitis by the casein number it is obvious that unsatisfactory results will be obtained if the milk samples are kept too long at room temperature. The rate of proteolysis naturally varies from sample to sample with differences in flora and enzyme content, and it is difficult to fix a time limit within which the sample should be analysed in order that the result may be considered applicable to the milk as it leaves the cow. It is clearly desirable to keep at a low temperature samples that have to await analysis, or to use a preservative, as described later.

Full details of the change in nitrogen distribution accompanying the reduction in casein number were obtained for milk samples numbers 3, 5 and 7, by a modification of the semi-micro method described in an earlier paper (2). These details are given in Table III. The reduction in casein number is shown to be due to the readiness with which casein is hydrolysed to proteose-peptone and non-protein nitrogen substances. The albumin and globulin are hydrolysed very slowly.

Table III. *Proteolysis in milk on keeping at* 16–18° C.

Sample no.	Nitrogen fraction as % of total N	Age of milk sample in hours: 0	24	48	96
3	Casein N	78·9	77·2	76·2	74·8
	Albumin + globulin N	12·2	17·6	17·3	12·1
	Proteose-peptone N	4·0			5·8
	Non-protein N	4·9	5·1	6·5	7·3
5	Casein N	77·4	77·0	76·4	75·7
	Albumin + globulin N	12·8	12·8	12·6	12·2
	Proteose-peptone N	3·6	3·9	4·5	5·3
	Non-protein N	6·2	6·3	6·5	6·8
7	Casein N	66·2	62·4	—	—
	Albumin + globulin N	19·4	19·4	—	—
	Proteose-peptone N	9·5	12·5	—	—
	Non-protein N	4·9	5·7	—	—

Effect of storage at 0–2° C., *and with preservatives at room temperature*

The effect on the casein number of keeping milk in a refrigerator at 0–2° C., and at room temperature (16–18° C.) with the following preservatives added, was next investigated:

Mercuric chloride, 0·1 g./l., i.e. 1 in 10,000.

Formaldehyde, 0·2 g./l., i.e. 1 in 5000.

Potassium dichromate, 2·0 g./l., i.e. 1 in 500.

Crystal violet, 0·125 g./l., i.e. 1 in 8000.

Sodium azide, 0·05 g./l., i.e. 1 in 20,000.

The results obtained with five different milk samples are recorded in Table IV; sample H was an infected sample.

Before the experiments with the preservatives were carried out it was considered possible that mercuric chloride and formaldehyde might interfere with the accurate determination of casein by rendering insoluble some of the albumin and globulin. The casein number of samples F, G, and H was determined after 3 hr. as it was realized that an initial increase of casein number due to the denaturation of soluble protein might not be apparent after 24 hr., owing to subsequent proteolysis. The results show occasional very small increases in casein number which are probably due to this denaturation.

The keeping of milk at a low temperature, or the addition to it of preservatives, retarded, but did not prevent, the hydrolysis of casein and the consequent

Table IV. *Effect on casein number of storage* (1) *at* 0–2° C. *and* (2) *with preservatives at* 16–18° C.

Treatment of milk sample	Casein no. Time in hours 3	24	48	66	96	168
Milk sample D, casein number 79·9						
16–18° C.	—	79·7	79·2	78·1	—	68·1
0–2° C.	—	79·8	79·4	79·2	—	78·4
Mercuric chloride	—	80·5	79·7	79·3	—	—*
Potassium dichromate	—	80·0	79·8	79·0	—	77·5
Formaldehyde	—	80·0	79·9	79·6	—	78·1
Crystal violet	—	79·6	79·1	76·1	—	—*
Milk sample E, casein number 79·0						
16–18° C.	—	78·4	77·4	—	66·9	—
0–2° C.	—	79·0	78·5	—	78·4	—
Mercuric chloride	—	79·2	78·1	—	76·5	—
Potassium dichromate	—	78·8	78·6	—	77·5	—
Crystal violet	—	78·5	77·9	—	70·1	—
Sodium azide	—	78·4	77·6	—	75·9	—
Milk sample F, casein number 79·6						
16–18° C.	79·6	79·4	75·3	—	—	—
0–2° C.	79·4	79·7	79·4	—	—	—
Mercuric chloride	80·0	80·0	79·4	—	—	—
Potassium dichromate	79·4	79·6	79·7	—	—	—
Formaldehyde	79·8	79·7	76·3	—	—	—
Milk sample G, casein number 79·2						
16–18° C.	78·9	78·7	71·7	—	—	—
0–2° C.	79·1	79·0	79·2	—	—	—
Mercuric chloride	79·1	79·4	79·0	—	—	—
Potassium dichromate	79·5	79·2	79·0	—	—	—
Forma.dehyde	79·4	79·2	79·1	—	—	—
Milk sample H, casein number 73·4						
16–18° C.	72·9	71·1	62·1	—	—	—
0–2° C.	73·4	72·5	72·3	—	—	—
Mercuric chloride	74·0	71·3	70·8	—	—	—
Potassium dichromate	73·8	72·7	70·7	—	—	—
Formaldehyde	73·3	72·3	70·8	—	—	—

* Sample coagulated.

reduction of the casein number. If milk samples for the diagnosis of mastitis by the casein number cannot be analysed early, storage at a temperature of 0–2° C., or the addition of 0·2 g. of potassium dichromate, 0·05 g. of formalin, or 0·01 g. of mercuric chloride per 100 ml. of milk at room temperature, will be satisfactory for about 24–48 hr. More definite conclusions cannot be drawn owing to the wide variation in the response of different samples.

SUMMARY

1. An investigation was made of the effect on the casein number of (*a*) advanced lactation, (*b*) storage of the milk sample at room temperature and in the refrigerator, and (*c*) storage of the sample at room temperature after the addition of the following preservatives: potassium dichromate, mercuric chloride, formaldehyde, crystal violet and sodium azide.

2. The casein number method of mastitis diagnosis is not applicable to cows in advanced lactation. It did not distinguish between infected and uninfected quarters, as all quarters in the drying-off stage produced milk with a number below normal. At the end of lactation the mean casein, soluble protein plus non-protein, and total nitrogen contents were 21, 86, and 36% greater respectively than when the cows were in full milk.

3. There was a decrease in the casein number of the milk samples on keeping at 16–18° C. or with preservatives owing to the hydrolysis of casein to proteose-peptone and non-protein nitrogenous substances. For the diagnosis of mastitis from the casein number, samples should not be kept at room temperature for more than a few hours before analysis. Keeping at 0–2° C., and at room temperature with the addition of 0·2, 0·05, and 0·01 g. potassium dichromate, formalin or mercuric chloride respectively per 100 ml. of milk, prevented significant reduction of the casein number for 24–48 hr.

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226. THE EFFECT OF COMMERCIAL DRYING AND EVAPORATION ON THE NUTRITIVE PROPERTIES OF MILK

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(With 1 Figure)

The experiments to be described here form a continuation of our work[1,2] on the effect of various commercial heat treatments on the nutritive value of milk. In the present series of experiments we have turned our attention to dried and evaporated milks. Thanks to the kindness of Messrs Aplin and Barrett, Ltd. we have been able to obtain from their Frome factory two types of dried milk and one of evaporated, all prepared simultaneously from one bulk of raw milk.

The preparation of the milks

The processing was carried out on 1 October 1937 and was as follows: 1500 gal. of mixed milk from Somerset herds of predominantly Shorthorn type were placed immediately after receipt at the dairy in a large storage tank. After thorough mixing a sample was withdrawn for analysis. Batches were then drawn off and submitted to the following processes:

(1) *Raw milk.* 17 gal. were withdrawn and of this 10 pints were put immediately into sterile thermos flasks and brought to the Institute at Reading the same evening. The remainder was cooled to 35° F., 50 pints were put into sterile thermos flasks and the rest into a churn. This milk was dispatched to Reading by rail and was received in the laboratory at 10 a.m. the following morning. There was some clotting in both batches, especially in the uncooled one.

(2) *Spray-dried milk.* 500 gal. of raw milk were passed over an open tinned copper forewarmer and then flash-heated in an internal tubular heater to 160° F. and held at this temperature for 1–4 min. Some of the tin was missing from the forewarmer and the milk was probably exposed in it to naked copper for about 20 sec. during which time the temperature was raised from 45 to 110° F. The milk was then concentrated at 110° F. in a Kestner climbing film evaporator. This process was completed in 10 min. when the milk reached a concentration of 45% total solids. Within 30 min. of concentration the milk was fed into a Kestner spray-drier with an inlet temperature of 320–329° F. and an outlet temperature of 203° F. For the bulk of the milk the time of passage through the drier was about 30 sec., variations were from about 5 sec. to about 15 min. (this latter figure applies to milk which unavoidably adhered to the sides of the drier). The milk powder was then put into 1 and $3\frac{3}{4}$ lb. tins and sealed at once in an atmosphere of 75% N_2 and 25% CO_2.

(3) *Roller-dried milk.* 50 gal. of raw milk were flash-heated to 160° F. as in the case of the spray-dried milk. The milk was then fed at this temperature on to Hatmaker rollers running at 45 lb. steam pressure and 13 rev./min. After milling it was put into tins and sealed as already described.

(4) *Evaporated milk.* 50 gal. were pre-heated to 190° F. and then evaporated in a small copper batch evaporator for 1 hr. at 120° F. After evaporation the milk was heated to 140° F. and homogenized at 2000 lb. pressure, filled into 6 and 12 oz. tins and sterilized in two batches. The first batch was heated to 240° F. within 20 min., maintained at this temperature for 17 min., rapidly cooled to 90° F. and shaken to break the casein clot. This batch showed a heavy casein clot which could not be broken up properly and the lactose was unduly caramelized. The second batch was much more satisfactory. In this case the sterilization temperature was raised to 240° F. within 15 min. This was maintained for a further 15 min. followed by rapid cooling to 90° F. It should be noted that no stabilizing agent ($NaHCO_3$) was added to the evaporated milk.

On receipt at the laboratory the milks were stored in a cool dark place until required for use.

PLAN OF THE EXPERIMENTS

The following were estimated by biological methods: (1) the biological value and digestibility of the proteins, (2) the vitamin B_1 content, and (3) the growth-promoting properties of the milks. In addition the following vitamins were measured in the milks by chemical or physical methods: (4) vitamin A and carotene, (5) riboflavin (lactoflavin) and (6) vitamin C.

In addition, other tests, which will be described in detail elsewhere, were carried out on these milks. These tests included a fluorimetric estimation of vitamin B_1 (cf. p. 279), a metabolic study of the availability of calcium in the spray-dried milk (Henry & Kon(3)) and an investigation of the effect of dried milk diets on the development of experimental lactose cataract in rats (Cashell and Kon(4)).

As soon as the milks were received at Shinfield the chemical tests were carried out both on the raw milk and on the dried and evaporated samples. These tests were repeated at intervals for a year on the dried and evaporated milks.

The biological tests were started within a week or two of the receipt of the milks. It was not possible to carry out a comparison with the original raw milk. In sections (2) and (3) studies on another raw milk were included. For this purpose evening and morning samples of bulk milk from this Institute were blended so that the resulting sample had the same fat content (3·8%) as the bulk milk which was used for drying and evaporation. The dried and evaporated milks used in these two tests were reconstituted so that they had the same fat content as the original raw milk.

EXPERIMENTAL

Reconstitution of the milks. The bulk sample of raw milk showed on analysis at Frome a fat content of 3·8%. On receipt at Reading it gave the same fat percentage and 12·53% total solids. The moisture contents of the two dried and of the evaporated milks were determined and it was calculated that the following amounts of the milks should be made up to 1 l. with distilled water in order to give a sample similar in composition to the original milk: spray-dried milk 131·2 g.; roller-dried milk 131·5 g.; evaporated milk 447·8 g.

The procedure was as follows: (i) *Spray-dried milk.* The required amount of dried milk was added to the bulk of the necessary water; this was well shaken and the solution made up to the final volume. (ii) *Roller-dried milk.* Owing to incomplete solubility the necessary

amount of dried milk was ground with some water in a mortar, made up to the required volume, stirred in a small butter churn for 1 min., then warmed to 30–40° C. to ensure complete solution. (iii) *Evaporated milk.* The milk was made up to the required volume with water, shaken and then stirred in the churn for 2 min. The second batch of milk was used for the feeding tests but some of the chemical and physical tests were carried out on both batches.

On analysis the reconstituted milks had the following composition:

	% fat	% total solids
Roller-dried milk	3·8	12·73
Spray-dried milk	3·8	12·74
Evaporated milk	3·8	12·51
The original raw milk contained	3·8	12·53

Biological tests

(1) *The biological values and digestibilities of the proteins (nitrogen) of the milks*

It has already been shown in this laboratory that though pasteurization has no deleterious effect on the proteins of milk (5), sterilization results in a lowering of the biological value by about 6% (6). In the present work tests were carried out on roller-dried, spray-dried and evaporated milks. It was not possible to carry out simultaneous tests on raw milk.

Experimental.

The biological value of the proteins of the milks was determined by the method of Mitchell (7) as modified by Mitchell & Carman (8). Four sets of three litter-mate female rats were placed, when 22–23 days old and weighing 42–49 g., in metabolic cages. The cages, vitamin additions, and the methods of collection and analysis of the excreta have been fully described (5). The same low egg-nitrogen and "nitrogen-free" diets were used. The experimental milks were mixed with the "nitrogen-free" diet and fed at a 7·9% level of protein (N × 6·38) intake. The two diets containing dried milks were prepared in bulk at the start of the experiment, but the evaporated milk diet was mixed freshly each day. Moisture determinations were made on the diets each week. Each milk was tested in turn on all rats in the course of the experiment by alternating the diets in three periods.

Results.

The results of the experiment are given in Tables I–V. Table I contains the analyses of the milks for nitrogen and moisture and Table II the composition and analyses of the experimental milk diets. Table III gives the individual nitrogen metabolism data. Table IV shows the mean figures for the biological values, the true digestibilities and the "protein values" obtained on each of the milks, while Table V gives a statistical treatment of the mean differences shown in Table IV. The statistical method used was that of the paired *t*-test of "Student" (9, 10) as described by White (11). When comparing two substances the results obtained on the same rat in different periods were used.

Table I. *Analyses of the milks*

Milk	% N	% moisture
Roller-dried	4·151	1·85
Spray-dried	4·148	1·62
Evaporated	1·214	71·24

Table II. *The composition and analyses of the experimental milk diets as fed*

	Spray-dried milk diet	Roller-dried milk diet	Evaporated milk diet
Butter-fat	12·03	12·03	7·11
Rice starch	36·10	36·10	21·32
Potato starch	7·08	7·08	4·18
Castor sugar (ground)	12·03	12·03	7·11
Salts (Steenbock 40)	3·54	3·54	2·09
Spray-dried milk	29·21	—	—
Roller-dried milk	—	29·21	—
Evaporated milk	—	—	58·19
% N	1·273	1·214	0·722
% moisture	7·47	7·50	45·47

In the absence of figures for raw milk no direct estimate of the effects of each of the heat treatments on the milk proteins is possible. The results can, however, be judged on a relative basis.

Table IV shows that the biological values of the milks varied from 84 for the spray-dried to 80 for the evaporated milk, but according to Table V this range is within the limits of experimental error. The true digestibility of evaporated milk, 91, is also lower than that of roller-dried milk, 93, and of spray-dried milk, 94. The latter difference is statistically significant. Finally, the "protein value" of the milks (Mitchell(12)) which combines both the biological value and digestibility is again lower for the evaporated milk by some 5% but no statistical significance attaches to this difference.

It will be noticed that a very low "protein value" (13·6) was obtained for the spray-dried milk in the case of rat no. 9 in the second milk period. Low figures for the biological value and true digestibility contributed to this. Apart from the fact that this animal showed a poor appetite during this period there is no justification for its exclusion. In any case its omission would not affect the significance of the differences.

Fairbanks & Mitchell(13) have carried out experiments on skim milk both in the liquid and dried forms. They obtained a biological value of 82 both for roller-dried ("choice commercial") and for spray-dried (pre-heated) milk. This figure compares well with the figures of 83 and 84 obtained by us now.

It should be pointed out, however, that on a previous occasion (14) we obtained for spray-dried pre-heated skim milk fed at a 6·9% level of protein intake a value of 90 which is as high as the values noted by Fairbanks & Mitchell(13) for liquid milk and for spray-dried milk which had not been pre-heated. Recently Sumner(15) obtained for dried skim milk a biological value of 85 and a true digestibility of 95.

Table III. *Nitrogen metabolism: individual data for 6-day periods*

Rat no.	Type of milk	Initial wt. g.	Final wt. g.	Diet intake* g.	N intake mg.	Faecal N mg.	Body N in faeces mg.	Food N in faeces mg.	Absorbed N mg.	N in urine mg.	Body N in urine mg.	Food N in urine mg.	Food N retained mg.	Biological value	True digestibility	"Protein value" of milk*
							Low egg-nitrogen diet									
1	—	49	54	36·00	—	81·4	2·26†	—	—	75·7	145·5‡	—	—	—	—	—
2	—	45	51	37·55	—	81·5	2·17†	—	—	69·6	145·0‡	—	—	—	—	—
3	—	47	55	38·43	—	74·9	1·95†	—	—	75·6	148·3‡	—	—	—	—	—
4	—	49	54	37·65	—	87·2	2·32†	—	—	73·9	142·2‡	—	—	—	—	—
5	—	47	51	33·30	—	89·4	2·69†	—	—	69·0	140·9‡	—	—	—	—	—
6	—	50	57	40·25	—	90·9	2·26†	—	—	70·5	130·6‡	—	—	—	—	—
7	—	45	51	40·49	—	94·0	2·32†	—	—	68·0	141·6‡	—	—	—	—	—
8	—	47	57	44·32	—	89·6	2·02†	—	—	73·5	141·4‡	—	—	—	—	—
9	—	48	56	43·41	—	98·1	2·26†	—	—	71·0	136·5‡	—	—	—	—	—
10	—	48	54	40·27	—	100·8	2·50†	—	—	78·3	153·6‡	—	—	—	—	—
11	—	46	51	39·32	—	102·7	2·61†	—	—	70·5	143·9‡	—	—	—	—	—
12§	—	40	41	24·24	—	93·0	3·84†	—	—	99·4	242·4‡	—	—	—	—	—
							First milk period									
1	Roller-dried	67	84	56·03	735·7	185·3	133·0	52·3	683·4	190·6	125·3	65·3	618·1	90·4	92·9	21·1
2	Spray-dried	54	63	36·18	497·6	108·1	78·3	29·8	467·8	145·0	93·0	52·0	415·8	88·9	94·0	21·1
3	Evaporated	65	70	38·11	503·8	117·0	78·4	38·6	465·2	166·7	101·2	65·5	399·7	85·9	92·3	20·0
4	Roller-dried	65	76	41·51	545·0	128·5	94·9	33·6	511·4	147·3	93·2	54·1	457·3	89·4	93·8	21·2
5	Spray-dried	62	73	41·13	565·7	101·5	104·2	0·0	565·7	155·6	88·3	67·3	498·4	88·1	100·0	22·1
6	Evaporated	68	78	46·26	611·5	161·1	106·5	54·6	556·9	181·2	91·3	89·9	467·0	83·9	91·1	19·1
7	Roller-dried	64	75	51·09	670·8	189·1	120·8	68·3	602·5	170·6	95·8	74·8	527·7	87·6	89·8	19·7
8	Spray-dried	71	86	54·56	750·4	173·4	109·8	63·6	686·8	177·9	103·9	74·0	612·8	89·2	91·5	20·7
9	Evaporated	67	71	37·71	498·4	163·7	84·6	79·1	419·3	174·6	88·9	85·7	333·6	79·6	84·1	16·6
10	Roller-dried	70	85	56·25	738·6	181·0	145·2	35·8	702·8	182·4	117·7	66·5	636·3	90·5	95·2	21·6
11	Spray-dried	66	79	50·82	699·0	171·8	127·5	44·3	654·7	154·7	98·9	55·8	598·9	91·5	93·7	21·5
12	Evaporated	50	61	39·55	522·8	130·9	95·4	35·5	487·3	129·4	77·6	51·8	435·5	89·4	93·2	20·9
							Second milk period									
1	Evaporated	89	92	45·81	605·6	177·4	113·9	63·5	542·1	249·2	132·7	116·5	425·6	78·5	89·5	17·3
2	Roller-dried	69	85	51·21	672·4	166·6	110·4	56·2	616·2	199·0	95·9	103·9	512·3	83·1	91·6	19·1
3	Spray-dried	76	92	53·78	739·7	152·5	116·2	36·3	703·4	248·7	125·4	123·3	580·1	82·5	95·1	19·7
4	Evaporated	83	95	51·34	678·7	151·3	115·9	35·4	643·3	243·0	106·9	136·1	507·2	78·8	94·8	18·7
5	Roller-dried	78	89	47·35	621·7	156·2	112·7	43·5	578·2	216·2	99·6	116·6	461·6	79·8	93·0	18·4
6	Spray-dried	87	100	58·89	810·0	202·4	138·2	64·2	745·8	245·1	112·2	132·9	612·9	82·2	92·1	18·9
7	Evaporated	78	91	54·73	723·5	179·3	131·8	47·5	676·0	253·3	112·4	140·9	535·1	79·2	93·4	18·4
8	Roller-dried	89	107	61·43	806·6	222·4	123·2	99·2	707·4	221·5	119·2	102·3	605·1	85·5	87·7	19·0
9	Spray-dried	74	77	38·32	527·1	195·6	85·4	110·2	416·9	215·6	92·0	123·6	293·3	70·4	79·1	13·6
10	Evaporated	91	96	49·79	658·2	195·0	132·3	62·7	595·5	250·3	139·3	111·0	484·5	81·4	90·5	18·1
11	Roller-dried	78	90	51·30	673·6	159·8	123·4	36·4	637·2	257·2	106·7	150·5	486·7	76·4	94·6	18·0
12	Spray-dried	68	77	47·09	647·7	165·0	113·6	51·4	596·3	219·3	96·4	122·9	473·4	79·4	92·1	18·1

12	Spray-dried	68	77	47·09	647·7	165·0	113·6	51·4	596·3	219·3	96·4	122·9	473·4	79·4	92·1	18·1
							Third milk period									
1	Spray-dried	97	115	60·10	826·7	176·5	156·1	20·4	806·3	270·7	154·7	116·0	690·3	85·6	97·5	20·8
2	Evaporated	93	102	51·73	683·8	153·4	111·0	42·4	641·4	245·2	111·9	133·3	508·1	79·2	93·8	18·6
3	Roller-dried	99	109	53·96	708·5	184·2	122·2	62·0	646·5	266·4	155·8	110·6	535·9	82·9	91·3	19·0
4	Spray-dried	102	110	50·88	699·8	161·2	116·4	44·8	655·0	293·8	115·5	178·3	476·7	72·8	93·6	16·9
5	Evaporated	96	107	52·38	692·4	178·6	116·8	61·8	630·6	256·4	109·7	146·7	483·9	76·7	91·1	17·4
6	Roller-dried	105	122	67·32	883·9	213·1	161·0	52·1	831·8	341·2	129·7	211·5	620·3	74·6	94·1	17·4
7	Spray-dried	102	123	69·39	954·4	201·2	170·1	31·1	923·3	272·1	144·1	128·0	795·3	86·1	96·7	20·8
8	Evaporated	119	127	62·11	820·9	232·6	124·0	108·6	712·3	326·2	137·4	188·8	523·5	73·5	86·8	15·9
9	Roller-dried	84	105	61·34	805·4	174·6	135·8	38·8	766·6	243·2	107·6	135·6	631·0	82·3	95·2	19·7
10	Spray-dried	102	124	64·70	889·9	193·0	176·8	16·2	873·7	243·8	164·5	79·3	794·4	90·9	98·2	17·4
11	Evaporated	104	110	52·40	692·7	191·3	120·6	70·7	622·0	256·2	126·9	129·3	492·7	79·2	89·8	17·6
12	Roller-dried	85	93	50·24	659·7	175·5	121·3	54·2	605·5	249·5	111·8	137·7	467·8	77·3	91·8	17·6
							Low egg-nitrogen diet									
1	—	124	124	58·98	—	159·8	2·71†	—	—	181·1	146·0‡	—	—	—	—	—
2	—	106	116	59·02	—	126·1	2·14†	—	—	115·3	103·9‡	—	—	—	—	—
3	—	110	113	49·81	—	118·0	2·37†	—	—	168·2	150·2‡	—	—	—	—	—
4	—	117	125	55·67	—	122·5	2·20†	—	—	118·4	97·9‡	—	—	—	—	—
5	—	112	128	63·88	—	132·7	2·08†	—	—	115·6	96·3‡	—	—	—	—	—
6	—	128	134	61·92	—	150·8	2·44†	—	—	141·7	108·2‡	—	—	—	—	—
7	—	129	130	56·94	—	142·0	2·49†	—	—	159·6	122·8‡	—	—	—	—	—
8	—	132	146	72·94	—	145·0	1·99†	—	—	141·4	101·7‡	—	—	—	—	—
9	—	114	119	56·21	—	123·6	2·20†	—	—	123·4	105·5‡	—	—	—	—	—
10	—	127	129	63·85	—	179·3	2·81†	—	—	182·7	142·7‡	—	—	—	—	—
11	—	112	117	57·21	—	125·6	2·20†	—	—	126·6	110·1‡	—	—	—	—	—
12	—	100	105	61·19	—	147·8	2·42†	—	—	122·7	119·1‡	—	—	—	—	—

* On the dry basis.

† These figures represent the metabolic faecal nitrogen per g. of food consumed and are used in estimating the body N in the faeces in the intermediate periods from the amounts of food consumed.

‡ These figures represent the endogenous urinary nitrogen per 100 g. of body weight and are used in estimating the body N in the urine in the intermediate periods from the average body weight.

§ The figures for body N in the faeces and urine of this rat are abnormally high owing to its poor growth and low food intake in this period. For this reason, in the subsequent calculations, the mean values of 2·31 mg. for body N in faeces and 142·7 mg. for body N in urine given by the remaining eleven rats were used.

Table IV. *Biological values, true digestibilities and protein values of the dried and evaporated milks*

	Spray-dried milk			Roller-dried milk			Evaporated milk		
Period	Biological value	True digestibility	Protein value	Biological value	True digestibility	Protein value	Biological value	True digestibility	Protein value
I	89·43	94·80	21·32	89·48	92·93	20·90	84·70	90·17	19·16
II	78·63	89·60	17·56	81·20	91·72	18·61	79·48	92·05	18·13
III	83·85	96·50	18·97	79·28	93·10	18·43	77·15	90·35	17·38
Mean	83·97	93·63	19·30	83·32	92·58	19·32	80·44	90·87	18·22

Table V. *Statistical significance of the mean differences in the biological values, true digestibilities and protein values of the dried and evaporated milks*

Difference tested	Numerical difference	Standard error of mean	P*
	Biological value		
Spray-dried – roller-dried	+0·65	±2·56	1: 1 Not significant
Spray-dried – evaporated	+3·53	±2·60	1: 5 Not significant
Roller-dried – evaporated	+2·88	±2·37	1: 4 Not significant
	True digestibility		
Spray-dried – roller-dried	+1·05	±1·77	1: 2 Not significant
Spray-dried – evaporated	+2·76	±1·22	1: 22 Significant
Roller-dried – evaporated	+1·71	±1·17	1: 6 Not significant
	Protein value		
Spray-dried – roller-dried	−0·02	±0·91	1: 1 Not significant
Spray-dried – evaporated	+1·08	±0·82	1: 5 Not significant
Roller-dried – evaporated	+1·10	±0·65	1: 8 Not significant

* P = Probability that a mean difference at least as great as the observed mean difference would have arisen by random sampling from a homogeneous population.

Swaminathan (16) reported, for the same type of milk, values of 89 and 83 at 5·3 and 10·4% levels of protein (N × 6·25) intake respectively. The true digestibility was 90 in both cases.

The figure for true digestibility now obtained for the spray-dried milk is rather less than those previously obtained by us for raw (5, 6) pasteurized (5), sterilized (6) and spray-dried milk (14). The figures for roller-dried and especially for the evaporated sample are definitely lower, though it is impossible to say whether this difference is statistically significant. Nevens & Shaw (17, 18) also fed various types of milk as the sole diet and found that the apparent digestibility of the proteins of milk was definitely lowered by roller- and spray-drying and by evaporation but found no difference between the various types of processed milks. The comparison between evaporated milk and the dried samples was, however, only indirect. Fairbanks & Mitchell (13), on the other hand, found only slight differences in true digestibility of raw and of choice commercial roller-dried or spray-dried preheated milk powder. Finally, Miyawaki *et al.* (19) found little difference in apparent digestibility between two samples of spray-dried milk and one sample of roller-dried milk but found that another sample of roller-dried milk was less digestible.

Our results suggest that evaporation is more injurious to the proteins of milk than spray- or roller-drying. The effects, with the exception of the decrease in true digestibility are not, however, sufficiently marked to show statistical significance.

(2) *The vitamin B_1 content of the milks*

Experiments in this laboratory have shown that commercial pasteurization of milk causes some loss of the vitamin B complex (Kon(20)) and that some 30% of the vitamin B_1 of milk is destroyed in the course of commercial sterilization (Henry & Kon(21)).

In the present experiments the vitamin B_1 content of the roller-dried, spray-dried and evaporated milk was determined by the method of Coward *et al.*(22). For comparison raw milk from this Institute was also tested.

Chemical (fluorimetric) estimations of the vitamin B_1 content of the different milks were also carried out (cf. note, p. 293).

Experimental.

Twenty-five litters of rats were weaned on to the stock diet and when weighing not less than 49 g., were placed on a vitamin B_1 deficient diet. The diet used was the routine diet deficient in the vitamin B complex(19), to 100 parts of which were added 25 parts of autoclaved yeast as a source of the vitamin B_2 complex. The autoclaved yeast was prepared by the method described by Coward *et al.*(21).

When the animals had become "run out", they were divided equally as regards sex and as evenly as possible as regards litter-mates into eleven groups each containing six bucks and six does. The international standard of vitamin B_1 (Kaolin adsorbate, no. V.B_1. 11) was fed at four daily levels, i.e. 10, 15, 20 and 30 mg. corresponding to 1, 1·5, 2 and 3 I.U. daily. For feeding, 1 part of the standard was mixed with 9 parts of icing sugar and moistened with water. The dried and evaporated milks were fed, reconstituted and each was given at levels of 8 and 12 ml. daily. The raw milk was fed at one level of 8 ml. daily. The animals were kept in cages in groups of three but the milks and the standard were fed singly in special cages. Double doses were given on Saturdays to avoid Sunday feeding. The test period lasted 3 weeks.

Results.

Table VI gives individual and mean figures for initial and final weights and for the gains in weight of the experimental animals. Curves of response were calculated for the international standard, and the vitamin B_1 contents of the milks were read from these curves as described by Coward *et al.*(22). Table VII gives the results both for separate and for pooled sexes.

As not infrequently happens in biological work, a paradoxical result was given by the male rats receiving the standard preparation, where the 1·5 I.U. group and the 2·0 I.U. group made identical gains. The results given by the females receiving the various levels of standard were much more satisfactory and, when the logarithm of the dose was plotted against the gain in weight, a straight line was obtained which passed through all four points. In certain cases, notably in the case of evaporated milk and of the lower level of the two dried milks, the gain in weight of the animals was less than that of the animals

Table VI. *The vitamin B_1 content of the milks. Individual and mean figures for initial and final weights and for the gains in weight of the experimental animals*

Rat no.	Weight of rat (g.) Initial	Final	Gain
1·0 I.U. Standard			
6797 ♀	75	104	29
6814 ♀	80	106	26
6873 ♀	82	102	20
6899 ♀	84	104	20
6946 ♀	82	105	23
7003 ♀	72	94	22
6832 ♂	83	128	45
6849 ♂	83	125	42
6887 ♂	81	142	61
6925 ♂	79	120	41
6971 ♂	77	100	23
7024 ♂	70	95	25
Mean ♀♀	79·2	102·5	23·3
Mean ♂♂	78·8	118·3	39·5
Mean ♀♀ and ♂♂	79·0	110·4	31·4
8 ml. roller-dried milk			
6828 ♀	88	128	40
6853 ♀	78	119	41
6889 ♀	80	110	30
6912 ♀	85	105	20
6954 ♀	80	120	40
7023 ♀	79	108	29
6801 ♂	71	103	32
6818 ♂	75	110	35
6877 ♂	85	110	25
6938 ♂	95	94	−1
6999 ♂	80	94	14
7009 ♂	78	90	12
Mean ♀♀	81·7	115·0	33·3
Mean ♂♂	80·7	100·2	19·5
Mean ♀♀ and ♂♂	81·2	107·6	26·4

Rat no.	Weight of rat (g.) Initial	Final	Gain
1·5 I.U. Standard			
6798 ♀	78	116	38
6815 ♀	68	106	38
6874 ♀	86	118	32
6900 ♀	69	102	33
6951 ♀	86	119	33
7004 ♀	76	107	31
6833 ♂	86	157	71
6888 ♂	76	135	59
6926 ♂	92	141	49
6972 ♂	71	108	37
7025 ♂	77	123	46
7041 ♂	97	143	46
	77·1	111·3	34·2
	83·2	134·5	51·3
	80·1	122·9	42·8
12 ml. roller-dried milk			
6829 ♀	81	132	51
6854 ♀	67	119	52
6890 ♀	83	126	43
6913 ♀	77	127	50
6994 ♀	68	107	39
7010 ♀	84	134	50
6802 ♂	77	137	60
6819 ♂	77	132	55
6878 ♂	78	122	44
6939 ♂	104	130	26
6955 ♂	84	140	56
7026 ♂	74	123	49
	76·7	124·2	47·5
	82·3	130·7	48·4
	79·5	127·4	47·9

Rat no.	Weight of rat (g.) Initial	Final	Gain
2·0 I.U. Standard			
6799 ♀	79	123	44
6851 ♀	61	102	41
6875 ♀	80	119	39
6952 ♀	71	115	44
7005 ♀	82	128	46
7021 ♀	70	107	37
6816 ♂	84	141	57
6834 ♂	87	142	55
6893 ♂	80	124	44
6903 ♂	79	137	58
6973 ♂	78	118	40
7033 ♂	83	136	53
	73·8	115·7	41·9
	81·8	133·0	51·2
	77·8	124·3	46·5
8 ml. evaporated milk			
6822 ♀	85	94	9
6843 ♀	89	100	11
6881 ♀	77	92	15
6919 ♀	77	90	13
6997 ♀	73	74	1
7013 ♀	77	89	12
6808 ♂	83	90	7
6857 ♂	77	90	13
6895 ♂	82	88	6
6958 ♂	85	124	39
7035 ♂	79	93	14
*7042 ♂	90	79	−11
	79·7	89·9	10·2
	82·7	94·0	11·3
	81·2	91·9	10·7

Rat no.	Weight of rat (g.) Initial	Final	Gain
3·0 I.U. Standard			
6800 ♀	78	131	53
6852 ♀	73	122	49
6876 ♀	80	127	47
6943 ♀	75	133	58
6953 ♀	78	131	53
7022 ♀	81	138	57
6817 ♂	93	149	56
6894 ♂	78	151	73
6904 ♂	74	147	73
6998 ♂	82	131	49
7008 ♂	79	138	59
7048 ♂	85	139	54
	77·5	130·3	52·8
	81·8	142·5	60·7
	79·6	136·4	56·8
12 ml. evaporated milk			
6823 ♀	81	108	27
6844 ♀	86	117	31
6882 ♀	80	99	19
6920 ♀	77	114	37
6966 ♀	72	98	26
7028 ♀	77	102	25
6809 ♂	89	125	36
6858 ♂	68	91	23
6896 ♂	83	114	31
6949 ♂	92	99	7
7000 ♂	78	91	13
7051 ♂	89	91	2
	78·8	106·3	27·5
	83·2	101·8	18·6
	81·0	104·0	23·0

Rat no.	Weight of rat (g.) Initial	Final	Gain
8 ml. spray-dried milk			
6820 ♀	77	112	35
6830 ♀	80	120	40
6891 ♀	79	100	21
6995 ♀	70	98	28
7011 ♀	82	117	35
7037 ♀	81	100	19
6803 ♂	81	121	40
6855 ♂	83	114	31
6879 ♂	78	102	24
6956 ♂	100	91	−9
7027 ♂	70	89	19
7050 ♂	85	94	9
	78·1	107·8	29·7
	82·8	101·8	19·0
	80·4	104·8	24·4
8 ml. raw milk			
6845 ♀	91	115	24
6859 ♀	82	111	29
6883 ♀	86	111	25
6921 ♀	77	104	27
6967 ♀	66	89	23
7029 ♀	85	111	26
6810 ♂	81	124	43
6824 ♂	94	123	29
6901 ♂	71	105	34
7001 ♂	79	103	24
7015 ♂	79	112	33
7043 ♂	89	114	25
	81·2	106·8	25·6
	82·2	113·5	31·3
	81·7	110·1	28·4

Rat no.	Weight of rat (g.) Initial	Final	Gain
12 ml. spray-dried milk			
6821 ♀	80	137	57
6831 ♀	80	136	56
6892 ♀	84	119	35
6915 ♀	79	119	40
7012 ♀	87	144	57
7038 ♀	75	114	39
6804 ♂	74	136	62
6856 ♂	75	118	43
6880 ♂	82	131	49
6942 ♂	83	146	63
7034 ♂	80	128	48
7040 ♂	99	134	35
	80·8	128·2	47·4
	82·2	132·2	50·0
	81·5	130·2	48·7

* Symptoms of beri-beri on the 21st day of the experiment.

Table VII. *The vitamin B_1 content of raw, spray-dried, roller-dried and evaporated milks expressed as international units per* 100 *ml. of reconstituted milk*

Type of milk	Daily level of intake ml.	Females	Males	Females and males
Raw	8	13·7 ± 1·3*	7·4†	10·8†
Spray-dried	8	15·9 ± 2·4*	3·9†	9·0†
	12	20·3 ± 2·4*	13·9 ± 4·8*	17·6
	Mean	18·1	13·9	17·6
Roller-dried	8	18·2 ± 2·6*	3·8†	9·8†
	12	20·5 ± 2·5*	12·7 ± 5·4	16·9
	Mean	19·4	12·7	16·9
Evaporated	8	7·5†	2·5†	4·8†
	12	9·8 ± 1·2*	2·4†	5·6†
	Mean	9·8	—	—

* Standard error of the mean. † By extrapolation—omitted from mean values.

receiving the lowest level of standard. Owing to the uncertainty of extrapolation, figures obtained from these groups were omitted from the averages. It is probably safer to place more reliance on the results obtained on the female rats but values obtained on both sexes are little different when only interpolated figures are considered.

The vitamin B_1 content of the roller- and spray-dried milks is virtually the same. The value, 18–19 I.U./100 ml., is rather higher than that obtained on raw milk (14 I.U./100 ml.), but it should be remembered that this milk was from a different source. It would appear, then, that spray- and roller-drying of milk caused little or no destruction of vitamin B_1. On the other hand, the vitamin B_1 content of the evaporated milk is only about half of the value obtained for the two dried samples. This difference is statistically highly significant. No doubt the much longer exposure to high temperature of this type of milk has contributed to the greater loss.

With the exception of Dutcher *et al.* (23) all earlier observers are agreed that evaporation causes some destruction of vitamin B_1. This was estimated by Samuels & Koch (24) at about one-fifth to one-sixth of the original potency of raw milk. According to Hartwell (25), Daniels & Brooks (26) and Daniels *et al.* (27) the loss, as far as could be judged by the less precise methods used at the time, was greater. The latest estimate of Daniels (28) puts the destruction at about 60%, but it seems that the milks were from different sources. There is less agreement regarding the relative values of vitamin B_1 in dried and evaporated milks. Thus, according to Hartwell (25) evaporation is much more injurious, but Daniels *et al.* (27) found roller-dried milk to be inferior to evaporated and spray-dried. In neither case were the milks made from the same bulk.

(3) *The growth-promoting properties of the milks*

A study was made of the growth-promoting properties of the dried and evaporated milks when they were fed, supplemented with minerals, as the sole diet to rats; this experiment was carried out from October to December. The growth of rats receiving these three experimental milks was compared with

that of rats receiving raw milk from this Institute blended to the same fat percentage (cf. p. 273). This milk was obtained from cows which were out by day and in at night. Their ration consisted of concentrates, kale and hay during October and November; in December the kale was replaced by mangels.

Experimental.

Litters were prepared for this experiment by the method described by Kohler *et al.*(29). Twelve sets of four male rats from eleven litters, weaned at 21 days, were placed in separate cages. They were allocated by lot to the four experimental milks. The milks were fed *ad libitum* according to the technique described by Bartlett *et al.*(30). The experiment lasted 8 weeks. The rats were then killed by coal gas, the body length measured (Donaldson(31)) and a post-mortem examination was carried out on each animal.

Results.

The average growth curves for the animals receiving the four types of milk are shown in Fig. 1. Individual data for gains in weight and for milk intakes are given in Table VIII and a statistical treatment of some of the differences shown there will be found in Table IX. One rat receiving evaporated milk, no. 7122, died after it had been on the experiment for a month. A post-mortem examination revealed marked gaseous distention of the large intestine, collapsed lungs and a displaced left kidney, both most probably due to the former cause. The mean figures for this group are therefore for eleven animals only. With the other milks two sets of mean figures are given: one includes all animals and the other excludes the litter-mates of the dead rat.

Fig. 1 and Table VIII show that the growth rate was very similar for all four groups of rats. The daily gain of nearly 3 g. is higher than that observed by us previously(30) in a similar experiment. The animals receiving the spray-dried and roller-dried milks made the best and those receiving the raw milk made the poorest gains in weight. The actual differences were not great and Table IX(*a*) shows that none of them was statistically significant. The body lengths of the four groups of rats were very similar and the differences can obviously have no statistical significance. There were, however, considerable differences in the milk intakes of the four groups of animals (Table VIII). The milk consumption of the rats receiving the evaporated milk was greatest, that of the raw milk rats lowest, and Table IX(*b*) shows that this difference in the consumptions of the raw and evaporated milks was statistically significant, but that the other differences in milk consumption were not.

It is not surprising, therefore, to find that, when the gains per 100 ml. milk intake are calculated, the best performance was made by the rats receiving raw milk and the poorest by the rats which were fed the evaporated milk. Differences in this respect between this last milk and the other three were in each case statistically significant. On the other hand, the gains per 100 ml. of the rats receiving raw, spray-dried and roller-dried milk were very similar and the differences had no statistical significance.

After reconstitution the evaporated milk was slightly lower in total solids than the two dried samples (p. 274). We allowed for this in a calculation

of gain in weight per gram total solids and found that the inferiority of the gains made by the evaporated milk rats still remained statistically significant.

One would expect that if, instead of the *ad libitum* feeding, the intakes of the milks were equalized, the rats receiving evaporated milk would have grown less well than the rats in the other groups.

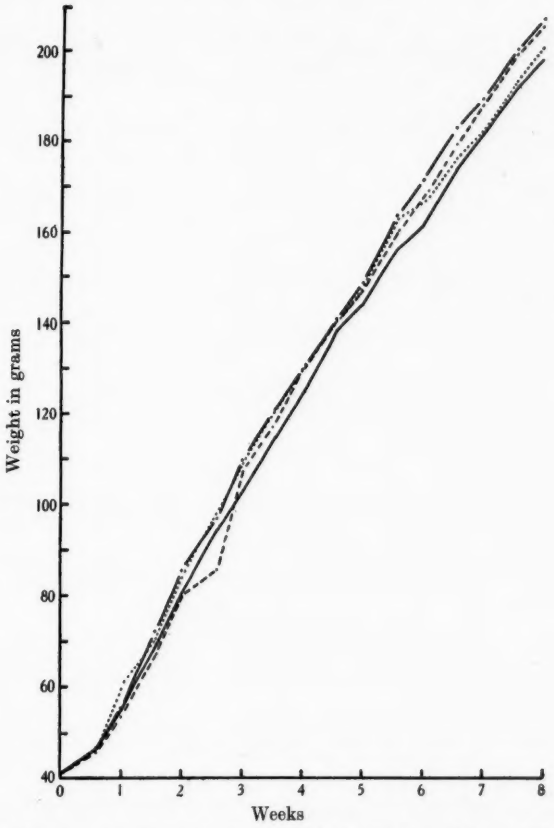

Fig. 1. Average growth curves (11 ♂♂ each) of animals receiving, *ad libitum*, the experimental milks supplemented with minerals.

——— Raw milk. ·– – –· Spray-dried milk.
·———· Roller-dried milk. ····· Evaporated milk.

It may be concluded from these experiments that, while evaporated milk is more palatable to rats, it is less well utilized than spray-dried or roller-dried milks. The reason for this lowered availability of evaporated milk is not clear. At such high levels of protein intake it is hardly likely to be due to the small changes in the biological value and digestibility of the proteins. Vitamin B_1 was also probably not at fault as, though evaporated milk contained only half as much of this factor as the other two (p. 281), yet an average intake of 86 ml.

Table VIII. *Individual and mean figures for gains in weight and milk intakes of the rats receiving, as an exclusive diet, the experimental milks supplemented with minerals*

Litter no.	Rat no.	Weight of rat (g.) Initial	Final	Gain	Gain/day g.	Milk intake ml.	g. gain/100 ml. milk intake	Body length mm.
		Raw milk						
I	7083	38	182	144	2·57	3571·5	4·03	200
II	7093	47	162	115	2·05	3741·5	3·07	201
III	7101	44	236	192	3·43	4553·0	4·22	212
IV	7110	40	140	100	1·79	3168·0	3·16	189
V*a*	7115	43	221	178	3·18	4226·0	4·21	210
V*b*	7121	42	247	205	3·66	4728·0	4·34	217
VI	7128	40	215	175	3·13	4588·0	3·81	210
VII	7134	44	227	183	3·27	4616·5	3·96	211
VIII	7143	43	233	190	3·39	4531·5	4·19	220
IX	7153	40	207	167	2·98	4390·0	3·80	209
X	7158	42	196	154	2·75	3912·0	3·94	209
XI	7180	38	153	115	2·05	3505·5	3·28	195
Mean		41·8	201·6	159·8	2·85	4127·6	3·83	206·9
Mean, omitting litter V*b*		41·7	197·4	155·7	2·78	4073·0	3·79	206·0
		Roller-dried milk						
I	7082	40	183	143	2·55	3772·0	3·79	200
II	7097	48	229	181	3·23	4607·5	3·93	211
III	7103	44	208	164	2·93	4356·0	3·76	206
IV	7109	37	126	89	1·59	3456·0	2·58	176
V*a*	7120	45	262	217	3·88	4923·0	4·41	210
V*b*	7118	42	218	176	3·14	4296·5	4·10	208
VI	7127	40	217	177	3·16	4685·5	3·78	204
VII	7135	40	196	156	2·79	4322·0	3·61	205
VIII	7148	42	256	214	3·82	5168·0	4·14	219
IX	7155	42	199	157	2·80	4262·5	3·68	205
X	7161	40	203	163	2·91	4273·0	3·81	206
XI	7179	40	196	156	2·79	4866·5	3·21	207
Mean		41·7	207·8	166·1	2·97	4415·7	3·73	204·8
Mean, omitting litter V*b*		41·6	206·8	165·2	2·95	4426·6	3·70	204·5
		Spray-dried milk						
	7081	38	186	148	2·64	3698·5	4·00	197
	7098	48	179	131	2·34	4044·0	3·24	203
	7104	44	219	175	3·13	4367·5	4·01	205
	7112	39	175	136	2·43	4168·5	3·26	200
	7116	46	261	215	3·84	4932·0	4·36	222
	7117	40	218	178	3·18	4756·5	3·74	210
	7129	40	174	134	2·39	4192·5	3·20	205
	7132	40	214	174	3·11	4692·0	3·71	207
	7142	45	270	225	4·02	5386·0	4·18	219
	7154	42	194	152	2·71	3956·0	3·84	205
	7160	36	180	144	2·57	4214·0	3·42	200
	7182	38	205	167	2·98	4623·5	3·61	202
		41·3	206·2	164·9	2·94	4419·3	3·71	206·3
		41·5	205·2	163·7	2·92	4388·6	3·71	205·9
		Evaporated milk						
	7080	41	223	182	3·25	4727·0	3·85	210
	7095	48	242	194	3·46	5426·5	3·58	216
	7105	42	148	106	1·89	3931·0	2·70	183
	7111	38	189	151	2·70	4470·0	3·38	202
	7119	44	272	228	4·07	6018·0	3·79	220
	7122	40	—	—	—	—	—	—
	7126	38	185	147	2·63	4615·5	3·18	203
	7136	40	172	132	2·36	4208·5	3·14	193
	7144	44	202	158	2·82	4718·5	3·35	211
	7152	37	156	119	2·13	3565·0	3·34	190
	7159	42	250	208	3·71	6062·0	3·43	214
	7181	39	161	122	2·18	4614·0	2·64	193
		41·2	200·0	158·8	2·84	4759·6	3·31	203·2

daily (Table VIII) supplied some 8 I.U. In any case a shortage of it would manifest itself by a decrease in calorie consumption which was not the case.

Table IX. *Statistical treatment of the differences observed in (a) the gains in weight, (b) the milk intakes and (c) the gains in weight per 100 ml. milk intake of the animals receiving the experimental milks*

Difference tested	Numerical difference	Standard error of mean	P*
	(*a*) Gains in weight (g.)		
Raw – spray dried	− 5·1	± 8·6	1: 2 Not significant
Raw – roller-dried	− 6·3	± 8·8	1: 2 Not significant
Raw – evaporated	− 3·1	± 16·5	1: 1 Not significant
Spray-dried – roller-dried	− 1·2	± 7·6	1: 1 Not significant
Spray-dried – evaporated	+ 4·9	± 14·7	1: 1 Not significant
Roller-dried – evaporated	+ 6·4	± 12·7	1: 2 Not significant
	(*b*) Milk intakes (ml.)		
Raw – spray-dried	− 291·7	±151·9	1: 12 Not significant
Raw – roller-dried	− 288·1	±153·3	1: 11 Not significant
Raw – evaporated	− 686·6	± 96·1	Over 1: 14, 700 significant
Spray-dried – roller-dried	+ 3·6	±111·0	1: 1 Not significant
Spray-dried – evaporated	− 371·0	±258·4	1: 6 Not significant
Roller-dried – evaporated	− 333·0	±247·6	1: 5 Not significant
	(*c*) g. gain in weight per 100 ml. milk intake		
Raw – spray-dried	+ 0·12	± 0·09	1: 5 Not significant
Raw – roller-dried	+ 0·10	± 0·11	1: 3 Not significant
Raw – evaporated	+ 0·48	± 0·16	1: 67 Significant
Spray-dried – roller-dried	− 0·02	± 0·12	1: 1 Not significant
Spray-dried – evaporated	+ 0·40	± 0·16	1: 36 Significant
Roller-dried – evaporated	+ 0·39	± 0·15	1: 41 Significant

* See footnote to Table V.

THE CHEMICAL EXPERIMENTS

(4) *The vitamin A and carotene content of the milks (milk fat)*

It has already been mentioned (p. 273) that the chemical tests included estimations on a sample of the raw milk from which the dried and evaporated samples were prepared.

Experimental.

It was found possible to obtain butter by churning from the spray- and roller-dried milks but not from evaporated milk. 2½ lb. of the dried milks were added to 17½ lb. of water, warmed to 150° F. and put twice through a separator. Roller-dried milk churned well but the spray-dried milk gave a poor yield of fat which was probably due to partial homogenization during spraying. As the evaporated milk was homogenized during manufacture it was not possible to separate and churn the fat from this milk; the fat was therefore extracted by the method described by Henry *et al.* (32). In view of differences in carotene content of churned and extracted fats (Henry *et al.* (32), these were obtained by both methods for the initial estimations from the raw milk and from the spray- and roller-dried milks. In subsequent estimations, which were carried out at intervals for a year, fat was obtained by churning from the two dried milks and by ether extraction from the evaporated milk.

The fats were examined for vitamin A and carotene by Lovibond Tintometer tests as described by Gillam *et. al.* (33).

Results.

The results of the experiment are shown in Tables X and XI. Table X gives the initial vitamin A and carotene contents of the milks expressed as Moore blue and yellow units respectively. It can be seen at once that none of the heat treatments had any deleterious effect on the vitamin A content of the milk.

Table X. *Initial vitamin A and carotene contents (Moore blue and yellow units) of extracted or churned fat from raw, spray-dried, roller-dried and evaporated milks*

	Extracted		Churned	
Type of milk	Vitamin A Moore blue units/g. fat	Carotene Moore yellow units/g. fat	Vitamin A Moore blue units/g. fat	Carotene Moore yellow units/g. fat
Raw	57·1	19·1	58·6	18·7
	57·1	19·3		
Spray-dried	60·0	20·8	60·0	17·3
	60·0	19·6		
Roller-dried	58·6	18·7	60·0	17·2
	60·0	20·0		
Evaporated 1st sample	60·0	17·1	—	—
,, 2nd sample	60·0	15·5	—	—

Neither method of drying (spray or roller) has caused a loss in the carotene content of the milk, but a very slight loss appears to have taken place with the evaporated milk.

Table XI gives a comparison of the vitamin A and carotene contents of the spray-dried, roller-dried and evaporated milks at intervals for a year. A fresh tin of each milk was opened for each estimation. Only the first batch of evaporated milk was used for these tests.

Table XI. *The effect of storage on the vitamin A and carotene content of spray-dried, roller-dried and evaporated milks*

		Spray-dried (churned)		Roller-dried (churned)		Evaporated (extracted)	
Date	Length of storage, months	Vitamin A Moore blue units/g. fat	Carotene Moore yellow units/g. fat	Vitamin A Moore blue units/g. fat	Carotene Moore yellow units/g. fat	Vitamin A Moore blue units/g. fat	Carotene Moore yellow units/g. fat
Oct. 1937	—	60·0	17·3	60·0	17·2	60·0	17·1
Nov. 1937	1	60·0	17·5	60·0	17·5	60·0	18·0
Jan. 1938	3	58·6	18·9	58·6	20·0	58·6	20·1
Apr. 1938	6	57·1	18·3	59·3	19·1	55·7	19·1
June 1938	8	57·1	14·5	57·1	15·5	58·6	13·8
Oct. 1938	12	60·0	16·5	59·4	16·0	60·7	18·9

It is at once evident from these figures that storage for a year has caused no loss in the vitamin A content of the fat of the three milks. The figures show only very slight variations throughout the year. The figures for carotene show greater variations from sample to sample and, in view of this, the slightly

lower values obtained for the last two samples of the spray- and roller-dried milks are probably not significant. The final carotene value obtained for the evaporated milk is higher than the initial and the variations obtained for these samples indicate that the figure given in Table X cannot be regarded as significantly lower than those obtained for the other milks.

The results of these experiments show that the vitamin A and carotene contents of milk are not affected when the milk is submitted to the processes of spray-drying, roller-drying or of evaporation. They also show that the vitamin A and carotene content of these milks remains unchanged after storage for a year.

(5) *Riboflavin (lactoflavin) content of the milks*

Experimental.

Riboflavin was measured fluoroscopically. Milks were prepared for this purpose by two methods, an alcoholic extraction method (method 1) based mainly on the technique described by Itter *et al.*(34) but using methyl alcohol in place of ethyl alcohol as extractant, and the precipitation method of Whitnah *et al.*(35) (method 2). The extraction method was applied to milk as follows: to about 10 g. of the dried milks or 20 g. of the evaporated milks were added 100 ml. of 95% methyl alcohol and 0·1 ml. of glacial acetic acid and the mixture was boiled under reflux in an atmosphere of nitrogen for 1 hr. After cooling and centrifuging, the solids were again refluxed with 50 ml. of 95% methyl alcohol and centrifuged. The residue was washed with two lots of 10 ml. of alcohol and the combined extracts were stored in the refrigerator over night. The lactose which crystallized out was removed by centrifuging and 1 ml. of $N/10$ $KMnO_4$ was added. After shaking for 1 min. the excess $KMnO_4$ was removed by H_2O_2(36). The extract was then evaporated *in vacuo* to about 40 and 10 ml. of the concentrated extract were used in each estimation. In both methods all operations were carried out as far as possible in red light. Riboflavin was measured by the intensity of its fluorescence in ultra-violet light by means of the fluorimeter described by Cohen(37). Chance's ultra-violet glass no. 14 was used for cutting off the visible radiations of the mercury vapour lamp and a Wratten 2*a* filter placed immediately before the selenium cell removed the ultra-violet, allowing only the fluorescent light to pass. In addition, a Wratten gelatine filter no. 12 was used to cut off any blue fluorescence due to substances other than riboflavin. A G.E.C. no. 1 selenium cell and a Cambridge d'Arsonval galvanometer were used. For reading, the solutions were placed in a quartz test tube. A solution of pure riboflavin (G.L. 5, Glaxo) was used for standardizing the instrument. Owing to difficulties arising from fluctuating voltage in the electricity supply at the Institute, the instrument was redesigned in April 1938 so that readings of the standard and unknown fluorescing solutions could be taken simultaneously. For this purpose the fluorimeter was divided into two compartments, one of which contained the standard solution and the other the unknown solution. The fluorescent light emitted by each solution was measured separately by two photo electric cells connected to two moving coil galvanometers and were read simultaneously on twin screens. The concentration of the standard solution was so adjusted that a deflexion of 100 mm. was obtained on the galvanometer scale.

Results.

The results are given in Table XII. It is not yet quite certain how reliable the fluorimetric test is. Some authorities (Fixsen & Roscoe(38); Ellinger(39)) believe that it gives much too low results. A comparison of the biological

findings of Kramer *et al.* (40) and of the fluorimetric tests of Whitnah *et al.* (40) suggest on the other hand fair agreement between the methods. Until a really reliable biological method for the estimation of riboflavin is available the problem will be a matter of speculation. In any case the usefulness of the fluorimetric test for comparative purposes as applied in the present paper cannot be doubted.

Table XII. *Riboflavin content of the original raw milk and of dried and evaporated samples after reconstitution expressed in* ($\frac{1}{1000}$ *mg.*) *per* 100 *ml.*

Raw milk (2 October 1937). Method 2. (*a*) Uncooled, 117 μg. of riboflavin per 100 ml. (*b*) Cooled, 109 μg. of riboflavin per 100 ml.

Date	Length of storage months	Spray-dried milk		Roller-dried milk		Evaporated milk no. 1		Evaporated milk no. 2	
		Method 1	Method 2	Method 1	Method 2	Method 1	Method 2	Method 1	Method 2
Oct. 1937	—	92	111	92	86	82	87	96	93
Nov. 1937	1	91	96	82	86	93	89	95	97
Feb. 1938	4	102	99	95	74	83	95	95	102
Apr. 1938	6	—	98	—	94	—	98	—	95
July 1938	9	—	97	—	84	—	96	—	94
Oct. 1938	12	—	89	—	88	—	98	—	96
Feb. 1939	16	—	86	—	87	—	94	—	94

As seen from Table XII the agreement between the two modifications of the fluorimetric method is quite satisfactory, especially when the initial difficulties in our laboratory are taken in consideration. On the whole the riboflavin content of the original raw milk and of the processed samples was definitely lower than values of 150–170 μg./100 ml. obtained by us for summer milks but was within the range 62–121 μg./100 ml. obtained by us for milk from stall-fed cows (Houston & Kon, unpublished work). The value for the raw milk is higher, with the exception of one test on spray-dried milk, than figures obtained for the processed milks, indicating a certain loss on heating. This is rather surprising, not only because of the heat stability of the flavin but also because in subsequent work (unpublished results of Houston & Kon) we found no loss in riboflavin content of milk after commercial pasteurization or sterilization. Moreover, samples of reconstituted spray-dried and evaporated skim milks made from one bulk of raw milk and recently tested by us gave values as high as the best summer values for our herd milk (over 170 μg./100 ml.). Though this is obviously only very indirect evidence, as the two batches of milks were from different sources, we feel that the question of destruction of riboflavin in the course of processing is still *sub judice* and would stress the remarkable stability of riboflavin in the dried and evaporated milks. There was hardly any change after 16 months' storage.

The vitamin C content of the milks

Experimental.

The ascorbic acid was measured in the milk by titration with 2:6-dichlorophenol-indophenol as described by Kon & Watson (42), both the reduced and reversibly oxidized forms being estimated. The milks were reconstituted before treatment with trichloroacetic

acid. Measurements on the processed samples were carried out for a year to determine the stability of ascorbic acid on storage.

Results.

The results are given in Table XIII. They show that in comparison with the sample of raw milk which was not exposed to light during cooling, all processed milks have suffered some loss of total vitamin C. This amounted to 20% in spray drying and to about 30% for the other two treatments. These values compare quite favourably with the loss of 21% in commercial pasteurization (Kon & Watson(43))—and of 43% in commercial sterilization (Henry & Kon(44)). The processed milks were sealed immediately after manufacture in metal containers under inert gas while pasteurized and sterilized milk was placed in glass bottles in which a certain amount of subsequent exposure to light could not be avoided. This may well explain why the apparently more drastic treatments of drying and evaporation were no more injurious. It should also be remembered that many of the operations were carried out *in vacuo* or anaerobically and most of them in the absence of light.

Table XIII. *The vitamin C content of the original raw milk and of dried and evaporated samples after reconstitution*

R., reduced. T., total ascorbic acid in mg./100 ml. T.%, total ascorbic acid expressed as % of total in uncooled raw milk.

		R.	T.
Raw milk:	(*a*) cooled*	1·13	1·55
	(*b*) uncooled	1·59	1·94

Date	Length of storage, months	Spray-dried milk			Roller-dried milk			Evaporated milk no. 1			Evaporated milk no. 2		
		R.	T.	T.%	R.	T.	T.%	R.	T.	T.%	R.	T.	T.%
Oct. 1937	—	1·09	1·55	80	0·77	1·33	69	0·77	1·43	74	0·48	1·24	64
Nov. 1937	1	1·00	1·58	81	0·78	1·28	66	0·77	1·60	83	0·77	1·28	66
Feb. 1938	4	0·98	1·58	81	0·76	1·20	62	0·68	1·10	57	0·46	0·98	51
Apr. 1938	7	1·03	1·58	81	0·77	1·17	60	0·61	0·98	51	0·29	0·75	39
July 1938	9	0·94	1·17	60	0·73	1·25	64	0·42	0·84	43	0·33	0·74	38
Oct. 1938	12	0·90	1·19	61	0·79	1·18	61	0·43	0·68	35	0·33	0·78	40

* The lower value for the cooled milk is probably due to exposure of the milk to light while running over the open cooler.

The stability of the vitamin C on storage was quite good, especially in the dried milks. In fact, there was no change in the spray-dried sample after 7 months' storage and virtually none in the roller-dried after a year. By that time the vitamin C content of the spray-dried milk had fallen to the same level as in the roller-dried, i.e. to about 60% of the amount originally present in the raw milk. The two evaporated samples lost vitamin C slowly but steadily in the course of storage, the final figures falling in one case to half the original value.

DISCUSSION

The findings embodied in our present paper and in our earlier reports(1,2) allow us to draw certain conclusions regarding the relative nutritional effects of drying and evaporation of milk as compared with pasteurization and sterilization. For ease of comparison these effects have been tabulated (Table XIV).

Table XIV. *Comparison of nutritional effects of various heat treatments and processing of milk*

Type of treatment	Change produced in relation to raw milk								Total growth-promoting value
	Vitamin A	Carotene	Vitamin B_1	Riboflavin	Vitamin C	Biological value of proteins	Digestibility of proteins	Availability Ca and P	
Pasteurization(1)	None	None	Some loss	—	Loss 20%	None	None	None	None
Spray-drying (present work)	None	None	?	Probably no change	Loss 20%	Possibly small lowering	Probably none	Probably none(3)	—
Roller-drying (present work)	None	None	?	Probably no change	Loss 30%	Possibly small lowering	Probably none	—	—
Sterilization(2)	None	Negligible	Loss 30%	None	Loss 40%	6% decrease	None	—	None
Evaporation (present work)	None	None	Loss 50% compared with roller- and spray-dried milk	Probably no change	Loss 30%	Probably slightly lower than in roller- and spray-dried milk	Slightly lower than in roller- and spray-dried milk	—	Less efficient growth per g. solids than on roller- and spray-dried milk

Figures in brackets indicate references to literature.

The comparisons are, of necessity, indirect. The only direct way would be to prepare all these milks from one bulk of raw milk and to compare them simultaneously. We have not been able to follow this counsel of perfection. On two points, the question of the relative loss of vitamin B_1 in drying and pasteurization and the question of the relative effect of these treatments on the biological value of the proteins, our own information is not sufficiently definite to allow us to voice an opinion. We are planning experiments which may allow us to answer the first point. Regarding the second, we found pasteurization to be without effect (5), while Fairbanks & Mitchell (13) noticed a loss after spray- and roller-drying. On the other hand, we (14) obtained a figure for the biological value of spray-dried skim milk numerically equal to that found by those authors (13) for raw liquid skim milk. In other respects there seems little to choose between commercial holder pasteurization and spray- and roller-drying in their immediate effects on the nutritive value of milk and we should like to endorse on the whole the view expressed seventeen years ago by the Committee on Nutritional Problems (Dried Milks) of the American Public Health Association (45) that dried milk is equal to pasteurized milk as regards the various factors of food value. Evaporated milk is definitely inferior to dried milk especially regarding the vitamin B_1 content, and to a lesser extent vitamin C and the digestibility of the proteins. The product appears to be in general less available (cf. p. 283). Of all types of milk tested by us this seems to have suffered most in the course of processing. It probably stands in the same relationship to dried milk as sterilized to pasteurized milk. It has not, however, the advantage of better keeping quality.

In assessing the nutritive value of dried milks their remarkable keeping qualities should be taken into consideration. Thus, after 16 months' storage at room temperature under inert gas our samples were still in perfect condition, riboflavin had suffered no loss, vitamin A and carotene were unchanged after a year and even such a labile substance as vitamin C was practically intact in the roller-dried sample and had decreased by only 25% in the spray-dried one.

In the evaluation of the relative merits of processed milks consideration of bacteriological purity, ease of reconstitution, palatability, bulk, and cost obviously play an outstanding rôle. They are, however, outside the scope of this investigation.

SUMMARY

1. Spray-dried (Kestner process), roller-dried and evaporated milks were prepared commercially from one batch of raw milk.

2. The biological value of the proteins (nitrogen), vitamin B_1 and the growth-promoting properties of the milks were measured by tests on rats.

3. Vitamin A and carotene, riboflavin and vitamin C were measured by physical and chemical methods at intervals in the course of storage for a year.

4. No significant difference was found between the biological values of the proteins (nitrogen) of the processed milks. The values ranged from 80 for evaporated milk to 84 for spray-dried milk.

5. The proteins (nitrogen) of evaporated milk were significantly less digestible (true digestibility 91) than those of spray-dried milk (true digestibility 94). The difference between either of those and roller-dried milk (true digestibility 93) was not significant.

6. The vitamin B_1 content of evaporated milk (9 I.U./100 ml. of reconstituted milk) was only half that of spray-dried (18 I.U./100 ml.) and of roller-dried (19 I.U./100 ml.). This difference was highly significant.

7. Reconstituted spray-dried, roller-dried and evaporated milks were fed *ad libitum*, supplemented with minerals, as the sole diet to groups of litter-mate male rats and produced in the course of 8 weeks practically identical gains in weight. The rats receiving evaporated milk consumed, however, more than those receiving the dried milks and, when the gains in weight per 100 ml. milk intake were calculated, the dried milks were found to be significantly superior.

8. Lovibond Tintometer estimations of vitamin A and of carotene showed that processing caused no loss of these substances. Moreover, they were not decreased after a year's storage of the processed milks.

9. There was no difference in the riboflavin content of the processed milks and the factor was not affected by 16 months' storage.

10. Spray-drying caused a 20% loss, roller-drying and evaporation a 30% loss in the original vitamin C content of milk. There was little further change in the roller-dried milk, but the vitamin C content of spray-dried milk declined slightly after 7 months' storage. There was a more definite and gradual loss in the evaporated milk.

We wish to express here our deep gratitude to Prof. E. Capstick who organized and supervised the preparation of the milks. Without his help this investigation could not have been carried out. Our best thanks are due to Mr A. L. Bacharach, Dr E. L. Smith and Mr W. J. Hurran of Glaxo Laboratories Ltd., for advice on the construction of the fluorimeter. We are indebted to Miss D. V. Dearden for the churning of the butters and to A. Wagstaff for fat analyses.

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Note added, 31 *May* 1939

Recent retests (biological method) of the B_1 content of spray-dried milk (stored 15 months) and roller dried and evaporated milk (stored 18 months) show a decline in potency of the former and little change in the two latter. The first (Kaolin adsorbate) I.S. B_1, was used in the first test and the second I.S. (crystalline B_1) in the retests, complicating comparison of results. Fluorimetric (cf. p. 279) did not at first agree with biological tests (Henry, Houston & Kon (1938) *Chem. Ind. Lond.* **57**, 974) and were not reported here. Recent modifications in the former (Houston & Kon (1939): *Nature, Lond.*, **143**, 558), measuring the various forms of B_1 in milk, show some loss of free B_1 on storage in all samples. The whole problem will be dealt with separately.

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227. THE RAPID DETERMINATION OF PEROXIDE VALUES FOR THE FAT IN MILK POWDERS

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If the peroxide value of a fat is to be a measure of its state of oxidation, it is essential that the estimation should be carried out as rapidly as possible and on a sample of fat which has been subjected only to the minimum amount of treatment necessary for its isolation. At the same time the solvents used throughout the test must be free from peroxides, for only when such precautions are taken can oxidation of the fat during the examination be reduced to a minimum.

With milk powders the extraction of the fat causes considerable difficulty, for it is found that soxhlet extraction with ether is only complete when the powder has previously been allowed to stand for some hours in a humid atmosphere so that its moisture content has been increased from about 2 to 10 or 11%. After the total fat has been extracted it is then possible to estimate the peroxide value by the method of Wheeler(1) which is founded on that of Lea(2). Wheeler dissolves the fat in a mixture of acetic acid (60%) and chloroform (40%) and allows this solution to act with potassium iodide, the iodine liberated by the peroxide being ultimately titrated with standard thiosulphate.

In developing the present method the chief objects in view were to avoid the use of peroxide-free ether (which is expensive, takes a considerable time to prepare and finally has to be evaporated from the fat), and to make the process as brief as possible by avoiding any necessity for soxhlet extraction and enhancement of the moisture content of the powder, which must usually precede it.

The method

10 g. of milk powder are weighed into a 100 ml. graduated flask and 50 ml. glacial acetic acid are added. To facilitate the dissolving of the fat, the mixture is warmed to 35° C. for 5 min. and during that time it is shaken at intervals. Chloroform is then added from a burette until the 100 ml. mark is reached, and during this addition the mixture is well-shaken several times. If it is desired to know as nearly as possible the total amount of solvent in the flask, the volume of chloroform which is required may be obtained from the burette. After a further vigorous shaking, the mixture is filtered rapidly through an ordinary filter funnel of at least 100 ml. capacity. An aliquot of the filtrate, as large as can conveniently be obtained, is then measured out, 1 ml. of a saturated solution of potassium iodide added, and the mixture shaken for 1 min. It is

then diluted with 100 ml. water and titrated with standard sodium thiosulphate, when normalities from 0·01 to 0·002 may be used. The whole determination is, of course, performed in duplicate.

It will be observed that in this procedure, extraction of the moistened powder with peroxide-free ether is avoided, and chloroform and acetic acid of the best quality are the only solvents used. The length of time required for the whole process from the opening of the can of powder till the peroxide value is known need not exceed 30 min. It is frequently recommended for the estimation of peroxide values that the test should be carried out in almost complete darkness so that further oxidation of the fat due to the effect of light may be avoided, but in the method just described, if the whole process is performed at a normal working speed, ordinary diffused daylight and artificial light do not affect the final reading.

The value obtained in this way is most readily expressed as ml. of standard thiosulphate per given aliquot of filtrate from 10 g. powder. This is sufficient where the method is being applied to comparative studies, such as the development of oxidation in milk powder on storage. The titration figure, however, can readily be converted to a figure roughly corresponding to the true peroxide value if the fat content of the powder has previously been determined. But to bring about this conversion, allowance must be made for the effective volume of the precipitate in the 100 ml. flask before filtering. In practice this appears to be 8 or 9 ml.

THE VALIDITY OF THE METHOD

This procedure for the estimation of peroxide values might have two main disadvantages. First, it is possible that the acetic acid-chloroform mixture might not extract all the fat from the powder, particularly if the latter were spray-dried. Secondly, even if the fat extraction were complete, the solvent mixture might remove enough material other than fat to interfere materially with the final value obtained. Experiments were therefore made to test the efficiency of different mixtures of chloroform and acetic acid as extractors of the fat under the conditions just outlined and also to find whether the peroxide value is affected by the presence of material other than fat which may be simultaneously extracted by the solvent. With regard to the first of these two points, Table I shows the results obtained with a spray-dried and a roller-dried powder known to contain 2·0 and 2·6% of moisture respectively. As the solvent was found to extract material other than fat, the true weight of the fat was obtained by re-extracting the residue from the acetic acid-chloroform mixture with light petroleum.

From the results which are recorded in Table I and which have been confirmed by further tests of a similar nature, the following conclusions may be drawn: (1) The maximum amount of fat is extracted by acetic acid-chloroform mixtures both from spray- and roller-dried powders provided the amount of acetic acid used for 5 g. powder is between 20 and 30 ml. Even with 15 ml.

Table I. *The extraction of fat from milk powder with acetic acid-chloroform mixtures*

Wt. of powder g.	Acetic acid added ml.	Ml. of $CHCl_3$ required to make the volume 50 ml.	Wt. in 25 ml. of filtrate A g.	A extracted with petroleum and reweighed	Fat percentage* Before petroleum extraction	Fat percentage* After petroleum extraction
			(*a*) Spray-dried powder			
5·100	None	Not recorded	0·1717	All soluble	6·2	6·2
5·211	10	,,	0·4593	0·4400	16·1	15·5
5·007	15	,,	0·8232	0·7237	30·1	26·5
5·014	20	,,	0·8388	0·7428	30·6	27·1
5·577	20	,,	0·8844	0·8072	29·0	26·5
5·198	20	,,	0·9221	0·7821	32·5	27·5
5·257	30	,,	0·9530	0·7837	33·2	27·3
			(*b*) Roller-dried powder			
5·017	10	35·5	0·6129	0·5808	22·2	21·1
5·004	15	30·6	0·8866	0·7294	32·3	26·6
5·004	20	25·8	0·9134	0·7329	33·5	26·8
5·011	20	25·7	0·8894	0·7412	32·4	27·0
5·002	30	15·7	1·0311	0·7488	37·7	27·4

(*a*) The fat content of this powder by direct ether extraction without raising the moisture content was as low as 10%. After raising the moisture content it became 27·4%, and by the Roese-Gottlieb method it was 26·1%.

(*b*) The fat extracted by ether without raising the moisture content of the powder was 21·2%, and after raising the moisture content it was 25·3%.

* Corrected for the approximate volume of the precipitate in the 50 ml. flask.

the extraction appears to be almost complete but with 10 ml. or less a partial extraction only is obtained. (2) Some material other than fat is simultaneously extracted, but this is insoluble in petroleum. It varies in amount, but appears to lie frequently between 5 and 10% of the original powder.

It seemed possible that this non-fatty material might cause either an increase or decrease in the apparent peroxide value of a fat. Tests were therefore made to investigate this question.

(1) If the material other than fat should cause an apparent increase in the peroxide value, it would be expected that skimmed milk powder would itself give a positive value, although it contains only a negligible amount of fat. Three skimmed milk powders were therefore extracted with the solvent mixture, and 50 ml. aliquots of the filtrate, which were found to contain appreciable quantities of non-fatty material, were shaken with potassium iodide solution. In no instance was any iodine liberated. This showed that no increase would normally be caused in the peroxide value (at any rate under the conditions of the present method of estimation) as a result of the extraction of non-fatty material with the acetic acid-chloroform mixture.

(2) In order to determine whether the peroxide value was decreased by the presence of the non-fatty material the following experiments were carried out:

(*a*) A sample of butter fat known to have a small peroxide value was weighed into three flasks and the value determined directly. Three further amounts were then weighed into 100 ml. flasks, each of which also contained

approximately 7 g. of skimmed milk powder. Again the peroxide value was determined by the method now under discussion. The results in terms of $N/100$ thiosulphate are recorded in Table II.

Table II. *The effect of the non-fatty material extracted by chloroform + acetic acid on the peroxide value of a fat*

Fat + pure acetic acid and chloroform

Wt. of fat g.	Titration ml. $N/100$ thiosulphate	Titration calculated for 2·5 g. ml.
2·418	0·39	0·40
2·423	0·36	0·37
2·458	0·39	0·40
	Mean	0·39

Fat mixed with spray-dried skimmed milk powder

Wt of fat g.	Wt of powder g.	Titration for 50 ml. filtrate ml. $N/100$ thiosulphate	Calculated titration for 2·5 g. fat ml.
2·483	6·915	0·20	0·37
2·514	6·855	0·18	0·33
2·460	6·950	0·18	0·34
		Mean	0·35

Although the titration figures shown in Table II are small, they are such as might very well be obtained in a typical peroxide value determination. They suggest that there is a tendency for the value to be somewhat reduced by the presence of this non-fatty material from the milk powder. A more complete series of tests was therefore undertaken.

(*b*) Two different samples of butter fat having fairly high peroxide values were used in the first two sets of tests. Different amounts of these fats were weighed into a series of flasks, pure acetic acid-chloroform mixture added, and the peroxide value determined in terms of $N/100$ thiosulphate. Parallel determinations were carried out with similar quantities weighed into another series of flasks, but the solvent mixtures used were the filtrates obtained from either a spray-dried skimmed milk or a roller-dried whole milk powder to which acetic acid and chloroform had been added, the method of extraction employed being that already outlined for the rapid determination of peroxide values.

A third set of tests was performed, using very much smaller amounts of a fat having a similar peroxide value together with the filtrate from a roller-dried skimmed milk. The results of these three experiments are recorded in Table III.

From the first and second set of experiments recorded in Table III it will be observed that the volume of $N/100$ sodium thiosulphate solution calculated to be required for 1 g. of fat when the filtrate from a milk powder was used as solvent, varied between 88 and 92% of that necessary when the solvent was pure chloroform and acetic acid. This held good when the actual volume of thiosulphate was varied between wide limits, i.e. from 9·0 to 0·9 ml. In the third set of experiments, where even smaller amounts of thiosulphate were

Table III. *The effect of the non-fatty material extracted by chloroform-acetic acid mixture on the peroxide value of a fat*

Wt. of fat g.	Titration (ml. *N*/100 thiosulphate)	Titration calculated for 1 g. fat ml.	Mean titration for 1 g. fat (A)
The fat + pure acetic acid and chloroform			
1·0424	9·12	8·76	8·51
1·0458	8·65	8·27	
0·4777	4·20	8·80	8·74
0·4780	4·15	8·69	
0·2156	2·10	9·74	9·48
0·2180	2·01	9·22	
0·0906	0·85	9·39	9·40
0·0988	0·93	9·42	
A second sample of fat + pure acetic acid and chloroform			
0·7600	6·92	9·10	9·03
0·7786	7·05	8·97	
0·2590	2·40	9·27	9·15
0·2570	2·32	9·03	
0·0955	0·89	9·32	9·30
0·0980	0·91	9·29	
A third sample of fat + pure acetic acid and chloroform			
0·0940	0·96	10·22	10·30
0·0722	0·75	10·39	
0·0554	0·55	9·93	9·95
0·0682	0·68	9·98	
0·0447	0·45	10·07	10·29
0·0447	0·47	10·52	
0·0298	0·32	10·74	10·40
0·0298	0·30	10·07	
0·0149	0·14	9·40	9·73
0·0149	0·15	10·06	
0·0060	0·05	8·33	8·33
0·0060	0·05	8·33	

Wt. of fat g.	Titration (ml. *N*/100 thiosulphate)	Titration calculated for 1 g. fat ml.	Mean titration for 1 g. fat (B)	$\frac{B \times 100}{A}$
The fat + the filtrate from a spray-dried skimmed milk powder				
1·0289	8·12	7·90	7·61	89·5
1·0108	7·40	7·32		
0·5184	3·97	7·66	7·98	91·3
0·5012	4·16	8·30		
0·2035	1·74	8·55	8·72	92·0
0·1790	1·59	8·89		
0·0987	0·80	8·11	8·59	91·4
0·0914	0·83	9·08		
			Mean	91·0%
The second sample of fat + the filtrate from a roller-dried whole milk powder				
0·6882	5·45	7·92	8·14	90·2
0·7362	6·15	8·36		
0·2666	2·18	8·18	8·08	88·4
0·2701	2·15	7·97		
0·0926	0·75	8·10	8·33	89·6
0·0981	0·84	8·57		
			Mean	89·4%
The third sample of fat + the filtrate from a roller-dried skimmed milk powder				
0·0880	0·75	8·53	8·77	85·2
0·0722	0·65	9·01		
0·0654	0·59	9·03	8·80	88·5
0·0688	0·59	8·58		
0·0455	0·40	8·80	9·02	87·7
0·0455	0·42	9·23		
0·0304	0·25	8·22	8·22	79·1
0·0304	0·25	8·22		
0·0152	0·14	9·22	9·22	94·7
0·0152	0·14	9·22		
0·0061	0·03	4·94	An average cannot reasonably be taken here as the titration is so very small	
0·0061	0·05	8·24		

required (0·05–1 ml.), the titration in the presence of the milk powder filtrate varied between 85 and 95% of that when pure solvents alone were used, with the exception of one low value of 79% which was obtained where the volume of thiosulphate required was only 0·25 ml. With abnormally low peroxide values the error in the titration (whether in the pure fat or in the fat *plus* extract) is so great that duplicate tests are unlikely to show close agreement (see last line of Table III).

It therefore seems reasonable to assume that as a general rule the peroxide value obtained by this rapid method will amount only to about 90% of the *true* peroxide value. But as the method has very great advantages in speed and ease of working, which are so desirable in a test of this kind, and as it dispenses entirely with prolonged methods of fat extraction, which may in themselves tend to produce erroneous peroxide values, the low estimation of the figure will not usually present any disadvantage. This is particularly true in experiments where the *relative* magnitude of the peroxide value is the main point at issue, as, for example, in estimating the approximate degree of oxidation in a milk powder possessing an "off" flavour, or in researches on the keeping qualities of whole milk powders during storage.

SUMMARY

1. A rapid method for determining the peroxide value of the fat contained in whole milk powders is described.

2. As estimated by this method the peroxide value is only about 90% of the true value, but in many researches where the method would be applied, it is the relative rather than the absolute peroxide value which is required. Consequently the disadvantage of a slightly low estimation is far outweighed by the rapidity of the method and the ease and reliability with which it can be performed.

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228. OXIDATION OF THE FAT OF BUTTER DURING COLD STORAGE

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Oxidation of the fat is one of the most serious causes of deterioration of butter submitted to long periods in cold storage. Salted butter, made from cream ripened by the action of micro-organisms, is known to be particularly susceptible to deterioration. Since the publication of the work of Rogers (1) it has generally been assumed that this susceptibility is due entirely to the acid formed during ripening. Rogers found that lactic, hydrochloric or acetic acids added to the creams used for the manufacture of salted butters caused a similar type of deterioration to that caused by ripening the cream.

The present author, working with unsalted butters, found, however, that the fat of butter made from cream which had been ripened to *p*H 5 showed considerable oxidation during 18 weeks' cold storage, whereas if the cream had been acidified with lactic acid, oxidation (and deterioration as shown by loss of grade) was very slight (2). The reason for the difference between Rogers's results for salted butters and the author's results for unsalted butters was not clear. Moreover the author's results were contrary to the view sometimes expressed (3) that unsalted butter from ripened cream has a better keeping quality than if made from unripened cream, due to the action of the starter organisms in large numbers in the ripened cream butters inhibiting oxidation by utilizing the available oxygen.

In view of the practical importance of determining whether starter organisms can influence the changes occurring in cold storage, an attempt has been made to define the separate influences of acidity, starter organisms and salt. During the course of the experiments it was found that the temperature of pasteurization of the cream had a marked effect on the results obtained. It was also found that butters made from different creams varied somewhat in their detailed behaviour on storage. When, therefore, a close comparison of different factors was desired the same cream was used for making all the butters. The variations between creams were, however, of minor importance and were never sufficient to invalidate the general conclusions regarding the influence of the other factors.

Tests for oxidation and relationship to butter grade

The degree of oxidation of the fat was measured for the most part by the determination of the fat aldehyde value (4). A small sample of the butter was melted by holding at 60° C. for 30 min. and 2 g. of the clear fat were taken for

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testing. The fat was not exposed at any time to a strong light. The peroxide value (5) was determined on a few samples and showed a fair correlation with the fat aldehyde value. The latter test, however, was found to be rather more sensitive in detecting a low degree of oxidation.

The butters were graded by experienced butter graders before and after storage. The grading naturally takes account of all off flavours and as a first step it appeared important to determine whether there was any direct relationship between the grade of the stored butter and oxidation as measured by the selected test. Table I shows the results of a number of determinations on butters, both salted and unsalted, made from creams pasteurized at 200° F.

Table I. *Relationship between fat-aldehyde values and grade of butter*

Grade after storage	Fat-aldehyde value	Mean
85–86	5·0, 3·6	4·3
86–87	4·2, 5·0, 8·3, 4·2, 1·9, 5·6, 6·3, 6·3, 5·0	5·2
87–88	6·3, 6·3, 8·3, 8·3, 8·3, 5·0, 2·8, 1·7, 2·5, 3·1, 3·1	5·1
88–89	6·3, 4·2, 5·0, 3·1, 2·8, 8·3, 4·2, 2·3	4·5
89–90	5·0, 7·1, 4·2, 2·8, 4·2	4·6
90–91	4·2, 2·8, 3·1, 0·8, 0·6, 0·2, 0·3, 0·1, 0·1, 0·2, 0·5	1·1
91–92	0, 0·03, 0, 0·13, 0·09, 0·13, 0·16, 0, 0, 0, 0, 0, 0·13, 0·08, 0, 0·4, 0·03, 0·7	0·15
92	0, 0, 0, 0, 0	0

While there was a considerable range in the fat aldehyde values corresponding to any one grade, if the mean results are taken there was invariably a low oxidation value when the grade was about 89 or better. Below this grade oxidation was very marked. With a badly deteriorated butter there is difficulty in deciding the exact number of points to award the butter. It is classed as a bad second grade and the exact score is not considered to be of much importance. Three butters which graded 90 points showed appreciably higher oxidation than the majority of the butters in the 90–91 group. Had they been graded 89·5 they would have fallen in the 89–90 group and have had typical fat-aldehyde values for the group. The butters which graded 85 were "fishy", which is a most objectionable fault, although not necessarily connected with a particularly high degree of fat oxidation.

The butters which graded 92 points after storage had lost on the average about one point in grade owing to "slight storage flavour". There is no evidence to show whether this was caused by a slight fat oxidation which the method was not sufficiently sensitive to detect or whether it was due to some other form of deterioration.

Method of manufacture and storage

The cream (containing about 38% fat) was flash pasteurized in a tandem flash pasteurizer and, unless it was to be ripened, was immediately cooled to about 48° F. and held overnight at that temperature. When the cream was to be ripened 5% of starter was added and the cream was held at 75° F. till the desired acidity was reached. It was then cooled to 48° F. and held overnight.

Most of the churnings were made in a small (3 l.) Alfa Laval churn and the butter was worked on a table worker, precautions being taken to avoid unnecessary exposure to strong light. The butters were washed twice. The salted butters contained 1·8–2·0% salt. On several occasions butters were also made, using an internal worker churn taking 300 lb. of cream, and butters from the same creams were made with the Alfa Laval churn, in order to determine any influence which the method of churning might have on keeping quality. Table II shows the fat-aldehyde values of the butter fats of a number of butters made from creams treated in various ways and churned in the large and small churns. The butters from the large churn were stored in 56 lb. boxes and the butters from the small churn, about 3 lb. each, were wrapped in parchfoil (aluminium foil covered on both sides with parchment).

Table II. *Comparison of fat-aldehyde values of butters prepared in large and small churns*

Large churn 56 lb. box		Small churn 3 lb. pats	
12 weeks' storage	25 weeks	12 weeks	25 weeks
0	0·05	0	0·1
0	Trace	0	0
3·6	6·3	3·1	6·3
3·1	5·0	3·5	6·0
6·3	8·3	6·2	9·0

There was no significant difference in the keeping quality of the butters from either churn.

After manufacture the butters were stored for 3 days at chill room temperature (35–40° F.) and were then placed in cold storage at 15° F. On removal from cold storage they were thawed out for 3 days at a temperature of approximately 60° F. and were then graded and examined in the laboratory.

The influence of lactic acid and ripening on oxidation

Four series of experiments were made in order to confirm the previously observed difference between acidifying and ripening the cream and to extend the observations to salted butters. In each series the cream was flash pasteurized at 200° F. One portion of the cream was used for the manufacture of sweet cream control butters, another portion was acidified with A.R. lactic acid to approximately *p*H 5 and another portion was ripened to approximately *p*H 5. Both salted and unsalted butters were made from each cream. The acidified creams, when held overnight at 48° F. for churning, increased slightly in *p*H, due, no doubt, to a slow attainment of equilibrium of the salts. The ripened creams fell slightly in *p*H when held at 48° F. If allowances were made for these changes, the acidifying and ripening could be controlled so that the *p*H at churning time was within $\pm 0{\cdot}1$ unit of the desired figure.

The fat-aldehyde values of the fats of these butters after storage for 10 and 26 weeks are shown in Table III.

Table III. *Fat-aldehyde values of unsalted and salted butters prepared from acidified, ripened and sweet creams*

		Acidified *p*H 5		Ripened *p*H 5		Sweet cream *p*H 6·6	
		10 weeks	26 weeks	10 weeks	26 weeks	10 weeks	26 weeks
Series 1	Unsalted	0	1·5	3·6	6·3	0	0
2	,,	0·1	2·3	3·1	6·0	0	0
3	,,	0	1·7	2·5	5·0	0	0
4	,,	0·1	2·3	3·0	6·3	0	0·05
Series 1	Salted	4·2	8·3	6·2	10·0	0	0·08
2	,,	3·1	5·0	8·3	8·3	0·03	0·06
3	,,	2·6	5·0	5·0	7·0	0	0·03
4	,,	3·2	5·5	5·5	8·5	0·03	0·06

The variations in values between the series show the differences that were found between butters made from different creams. There is a variation in susceptibility to oxidation but this is not great in comparison with the influence of the factors being studied.

The results show that with unsalted butters made from acidified cream there was very little oxidation after 10 weeks' storage but after 26 weeks there was quite definite oxidation. With the unsalted butters made from cream ripened to the same *p*H, oxidation was definite in 10 weeks and severe in 26 weeks. With the salted butters also, ripening caused a greater oxidation than acidifying. However, the salted acidified butters oxidized quite definitely in 10 weeks in striking contrast to the unsalted acidified butters. There was slight but apparently significant oxidation in the salted sweet cream controls. This table illustrates the results found in all the experiments, i.e. that the oxidation of the fat was greater with salted butters than with similarly treated unsalted butters, and greater with ripened cream butters than with acidified cream butters.

The effects of acidity, ripening, salt, and time are shown in more detail in Table IV, which gives the fat-aldehyde values for two series of butters. The cream for the acidified butters was all brought to *p*H 5 with lactic acid after pasteurizing at 200° F. and was then neutralized to the required *p*H with sodium hydroxide. All the butters therefore contained the same lactate concentration irrespective of *p*H. The cream for the ripened butters was pasteurized, ripened to *p*H 5 and neutralized, before churning, to the required *p*H with sodium hydroxide without subsequent pasteurization. These butters, therefore, had the same population of starter organisms and the same lactate concentration although differing in *p*H.

Table IV confirms the results given in Table III but shows some additional points of interest. At *p*H 5·7 there was generally appreciable oxidation but not to the same extent as at *p*H 5. The ripened cream butters, both salted and unsalted, when neutralized to *p*H 6·6 showed definite oxidation after 20 weeks' storage, indicating the presence of some oxidizing system which can function at *p*H 6·6 but which is favoured by higher acidities. The butters made from cream which had been acidified with lactic acid and then neutralized, oxidized

Table IV. *Effects of acidity, ripening, salt content and time on the fat-aldehyde values*

		Unsalted		Salted	
	*p*H	Series 1	Series 2	Series 1	Series 2
7 weeks' storage:					
Sweet cream control	6·6	0	0	0·03	0·03
Lactic acid added	6·6	0	0	0·03	0·03
	5·7	0	0	0·4	0·5
	5·0	0	0	1·9	1·9
Ripened	6·6	0·1	0·2	0·2	0·2
	5·7	0·6	0·8	1·4	1·0
	5·0	1·5	1·8	3·6	3·0
20 weeks' storage:					
Sweet cream control	6·6	0·05	0	0·1	0·2
Lactic acid added	6·6	0	0·05	0·1	0·2
	5·7	0·3	0·5	0·6	1·0
	5·0	2·1	2·7	3·0	3·5
Ripened	6·6	1·2	1·0	1·5	1·5
	5·7	1·8	1·3	2·3	3·1
	5·0	3·5	3·0	5·6	6·3

no more than the sweet cream controls, indicating that the lactate ion has no pro-oxidative action.

The influence of pasteurization temperature

The temperature of pasteurization was found to have a marked influence on the oxidation of the acid butters; the effect on the sweet cream butters was definite but not so marked. Table V gives the results of an examination of a series of sweet cream butters after 26 weeks' storage. The acid value was determined by titrating 20 g. of the filtered butter fat in 50 c.c. of hot alcohol with $N/10$ sodium hydroxide and is expressed as mg. KOH per 100 g. of fat.

Table V. *Influence of pasteurization temperature on fat-aldehyde value, and on grade and acid value before and after storage*

Pasteurizing temp. ° F.	Fat-aldehyde value	Grade before storage	Grade after storage	Acid value before storage	Acid value after storage
			Unsalted		
150	0·2	93·5	89	0·31	0·73
175	0·03	93·5	91	0·20	0·20
200	0	93·5	91·5	0·20	0·20
			Salted		
150	0·8	93	90·5	0·32	0·42
175	0·13	93·5	91·5	0·22	0·22
200	0·08	93	91·5	0·22	0·22

Table V shows that the butters made from cream pasteurized at 150° F. have oxidized appreciably more than those made from cream pasteurized at 200° F. However, the deterioration, as judged by grade, is much greater than the fat-aldehyde values would indicate. This additional deterioration is probably due to the action of lipase not destroyed by the low-temperature pasteurization, as is indicated by the increase in acid value of the fat during

storage. The deterioration is not so great with the salted butter, indicating inhibition of lipolytic action by salt.

Flash pasteurization at 150° F. is admittedly inefficient from the point of view of bacterial destruction although it destroys over 90% of the viable organisms. It is probable that the small bacterial population remaining after such pasteurization would not influence the deterioration of the butter when held at 15° F.

Table VI shows the fat-aldehyde values for butters made from ripened and acidified creams pasteurized at different temperatures.

Table VI. *Influence of different pasteurization temperatures on fat-aldehyde values of butters made from ripened and acidified creams*

Pasteurizing temp. ° F.	Sweet cream butters	Ripened cream butters *p*H 5	Acidified cream butters *p*H 5
		Unsalted	
10 weeks' storage:			
150	0·12	5·0	3·6
175	0·03	4·2	1·5
200	0	3·1	0·1
		Salted	
150	0·3	8·3	8·3
175	0·19	8·3	5·0
200	0	6·2	4·2
		Unsalted	
26 weeks' storage:			
150	0·2	7·0	5·0
175	0·03	6·5	4·2
200	0	5·0	2·8
		Salted	
150	0·8	10·0	8·3
175	0·13	8·3	7·3
200	0·08	6·3	5·3

The ripened cream butters pasteurized at 150° F. were oxidized more readily than those pasteurized at 200° F. The effect with the acidified butters, particularly the unsalted ones, is even more striking. After storage for 10 weeks those pasteurized at 200° F. showed negligible oxidation, whereas those pasteurized at 150° F. showed severe oxidation.

THE INFLUENCE OF SERUM ACIDITY ON BUTTER FAT ACIDITY

Briggs(6) found that lactic acid dissolved in butter fat hastened the oxidation of the fat at 100° C. It has been claimed (Davies(7)) that in butters from ripened cream the lactic acid dissolves in the fat and causes oxidation. It is most unlikely, however, that this could occur as lactic acid (dissociation constant $1{\cdot}38 \times 10^{-4}$) at *p*H 5 would be present to the extent of about 98% as a salt. The acid values of the fats of a number of butters which were stored for 26 weeks were measured before and after storage (Table VII). The cream for all these butters was pasteurized at 200° F.

Table VII. *Effects of storage on acid values*

	Sweet cream butter		Ripened (*p*H 5) cream butters		Acidified (*p*H 5) cream butters	
	Before storage	After storage	Before storage	After storage	Before storage	After storage
1. Unsalted	0·75	0·70	0·70	0·75	0·75	0·70
2. Unsalted	0·90	0·90	0·85	0·90	0·90	0·85
3. Salted	0·80	0·85	0·85	0·80	0·80	0·80
4. Salted	0·85	0·85	0·85	0·85	0·85	0·85

The fats of the butters with serum acidities of *p*H 5 (due to ripening or acidifying) had the same acid values as the fats of the corresponding sweet cream butters. The results showed no change in acid values after storage of the butters.

The influence of diacetyl and acetoin on fat oxidation

Barnicoat(8) found that the addition of diacetyl to sweet cream butters did not promote their deterioration in cold storage, although it had been claimed that diacetyl acted as a pro-oxidant in butter. In view of the difference found between ripened and acidified cream butters it was considered possible that at a low *p*H the diacetyl or acetoin formed by the starter organisms might function as a catalyst for the oxidation.

Three series of butters were prepared. The first series of butters was made from sweet cream and from portions of the same cream acidified with lactic acid to *p*H 5·7 and 5·0, with and without the addition of diacetyl. In the second series acetoin and in the third series both diacetyl and acetoin were substituted for the diacetyl. (These butters were made before the importance of pasteurization temperature was realized and were made from cream pasteurized at 145° F. for 30 min. There was greater oxidation of the fat of the butters acidified with lactic acid than would have occurred if the pasteurization had been done at 200° F.) Table VIII gives the results of the examination of the butters after 15 weeks' storage.

Table VIII. *Fat-aldehyde values after 15 weeks' storage*

Diacetyl or acetoin added to cream before churning	Sweet cream		*p*H 5·7		*p*H 5·0	
	Unsalted	Salted	Unsalted	Salted	Unsalted	Salted
Nil	0·2	0·4	1·2	1·7	2·3	4·9
6 p.p.m. diacetyl	0·2	0·4	1·3	1·6	2·3	4·9
Nil	0·1	0·2	1·1	1·4	1·7	4·2
100 p.p.m. acetoin	0·1	0·2	1·1	1·4	1·8	4·2
Nil	0·1	0·3	1·3	1·5	2·2	3·1
6 p.p.m. diacetyl + 100 p.p.m. acetoin	0·1	0·3	1·2	1·6	2·3	3·3

The diacetyl (6 parts per million) and acetoin (100 parts per million) were added to the creams immediately before churning. The proportions added were somewhat higher than occurred in creams ripened to *p*H 5.

The results show quite definitely that neither diacetyl, acetoin, nor mixtures of the two influence the oxidation of the fat at acidities between *p*H 6·6 and 5·0.

THE INFLUENCE OF STEAMED MILK ON THE OXIDATION

Ritter (9) found that butters made from cream pasteurized at 90° C. did not develop fishiness during storage, whereas butters made from creams pasteurized at a lower temperature became fishy. To explain this result Ritter postulates that the high temperature pasteurization produces an anti-oxidant in the cream. It was considered that if an antioxidant were really formed by heat it should be possible to demonstrate its presence by adding to cream, pasteurized at a low temperature, a proportion of milk which had been subjected to higher heat treatment. If an anti-oxidant were formed, butter made from this mixture should oxidize less readily than butter made from the same cream and milk when both were pasteurized at a low temperature. It was considered that the greater oxidation of ripened cream butters as compared with acidified cream butters might be due to the destruction of the assumed anti-oxidant by the process of ripening. The experiments were designed to give some information on this point.

Table IX gives the methods of treatment of the creams and the fat-aldehyde values of the butter fats after 20 weeks' storage.

Table IX. *Effect of products of heated milk on the fat-aldehyde values in stored butters*

	Unsalted	Salted
1. Cream + 20% raw milk pasteurized together at 200° F.	0	0·1
2. Cream + 20% raw milk pasteurized together at 145° F.	0·1	0·2
3. Cream + 20% raw milk pasteurized together at 145° F. acidified to *p*H 5·2	2·2	2·8
4. Cream pasteurized at 145° F. + 20% steamed milk	0·1	0·1
5. Cream pasteurized at 145° F. + 20% starter made from same steamed milk *p*H 5·2	3·2	4·2
6. Cream pasteurized at 145° F. + 20% steamed milk acidified to *p*H 5·2	2·2	2·8

The steamed milk was prepared by holding the milk for 30 min. at 212° F. The results show that the addition of 20% of such steamed milk had no influence on the oxidation of the butter fat induced by acidity when the cream had been pasteurized at a low temperature (145° F. for 30 min.). The presence of starter organisms caused increased oxidation, as had been found in previous experiments.

DISCUSSION OF RESULTS

The results show that in butter, low *p*H, the presence of starter organisms, low pasteurizing temperature, and salt all separately favour the oxidation of the fat at cold-storage temperatures.

The oxidation that occurs in the presence of starter organisms, even when the acidity formed by them was neutralized before churning, demonstrates the presence of some fat oxidizing system in the ripened cream. This might be some product of bacterial metabolism acting as a pro-oxidant; if so, the

product is certainly not diacetyl or acetoin. The good keeping quality of butter made from ripened cream which has been neutralized and then pasteurized is well known and certain experiments not quoted in detail here have shown that such butters were no more markedly oxidized during cold storage than sweet cream control butters. It follows that if a pro-oxidant is formed during ripening it is destroyed by the pasteurizing process. The results appear to be most readily explained by assuming that the lactic streptococci of the starter contain a fat-oxidizing enzyme which is most active at low *p*H and high salt concentration. During the experiments several mixed starters (*Streptococcus cremoris* and *Betacocci*) and a single strain of *Str. cremoris* were used and all caused the increased oxidation as compared with simply acidifying the cream. The effect, therefore, is not peculiar to any particular starter.

The comparatively rapid oxidation of acidified cream butters pasteurized at low temperatures would also indicate either a fat-oxidizing system in the raw cream which is destroyed by heat or the production of an anti-oxidant by heat. The experiments with steamed milk indicate that the former alternative is more probable. It might be objected that the anti-oxidant could be formed from the fat phase of the cream during pasteurization rather than the skim milk phase. This would be extremely improbable in view of the rapid increase in susceptibility to oxidation of fats with rising temperatures. Kende (10), who studied oxidized flavours in milk, found it necessary to postulate a fat-oxidizing enzyme "oleinase" which was not destroyed by low-temperature pasteurization. If the same enzyme is responsible for the oxidation of the butter fats its action is favoured by low *p*H and high salt concentration.

It is significant that Lea (11) and Banks (12) have demonstrated the presence of a fat-oxidizing system in the fatty tissue and muscle of the pig and the herring, the activity of the enzyme being particularly high between *p*H 4 and 5 and in the presence of a high concentration of salt.

Summary

The oxidation of the fat of butter during cold storage has been studied by measuring the fat-aldehyde value of the fat. A direct relationship was found between this value and the grade after storage.

It was found that acidity, starter organisms, salt and low-pasteurization temperatures each favour the oxidation. Neither diacetyl nor acetoin influence the oxidation.

The results indicate the presence in ripened pasteurized cream and in unripened raw cream of a fat-oxidizing enzyme which is most active at low *p*H values (about 5) and high salt concentrations.

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229. BACTERIOLOGICAL TESTING OF BUTTER. NEW AND SIMPLIFIED ROUTINE METHODS

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(With 1 Plate and 3 Figures)

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Introduction

Just as milk and cream are readily spoiled by the presence of certain types of bacteria, so one would expect butter to be similarly affected. Even with primitive methods of manufacture, however, butter has been found to keep in a reasonably edible condition far longer than milk or cream. Two reasons for this can be given. First, the majority of the bacteria from the cream are removed with the butter-milk, leaving only about 10% in the finished product. Second, the structure of butter, especially when well made, renders it an unfavourable medium for bacterial development. Again, when salt is added the growth of bacteria is greatly restricted. These factors materially diminish the influence of bacteria in spoiling butter even under the most insanitary conditions (such as have occurred in the distant past). With the advent of pasteurization of the cream, bacterial spoilage of butter is still less to be expected under modern conditions. This in part explains the failure of many attempts which have been made to relate the quality of butter shortly after manufacture to its bacterial content. At the same time the cream after pasteurization may easily be recontaminated by passage over coolers and through pipes, by storage in large vats, and during subsequent churning. Consequently, although the finished butter should contain few bacteria, large numbers may often be present. In New Zealand a knowledge of the bacterial content of butter soon after manufacture is important not so much in relation to the quality or grading of the

butter as to the hygienic conditions within the factory. By the time the butter reaches the consumer in England the relation of the bacterial count to the quality may be more important, especially in order to indicate unsatisfactory conditions associated with transit and storage since manufacture.

Apart from indicating the total bacterial content of butter, testing methods should also provide some information about the numbers of different types of organisms which are present. During the last 10 or 15 years special emphasis has been placed upon the yeast and mould content of butter, not so much because these organisms are likely to damage the butter, but because they are easily killed at comparatively low temperatures. Yeasts readily multiply in any deposit of sour-milk residues concealed about the plant, so that their presence in large numbers indicates that some part of the factory equipment is not being properly washed and sterilized. Coliform bacteria often give rise to objectionable flavours in milk and cream, but the literature contains few references to defects definitely attributable to their presence in butter. According to Hammer & Yale(1) the *Aerobacter* species are more harmful to butter than *Escherichia*. Like yeasts and moulds, however, they are easily killed at moderate temperatures and so provide a bacterial index of sanitation.

The studies of American workers (especially at New York and Iowa Stations) have shown that bacteria commonly present, chiefly micrococci, are much more difficult to kill than yeasts and coliform organisms. The present authors have therefore described them as "heat-resistant organisms". But even these bacteria may be killed by very hot water or steam, so that their presence in large numbers, especially in the absence of yeasts, may be taken to indicate that factory-cleaning operations have not been thorough. The investigations of Robertson(2) upon the flora of dairy utensils have shown certain yellow types to be common among the bacteria found to resist the prolonged application of various chlorine sterilizing solutions. Such solutions are therefore not to be recommended in dairy-factory practice as substitutes for hot soda solutions followed by ample boiling water and steam.

Two other types of organisms are worth mentioning because of the damage they may do to the butter. First, lipolytic or fat-splitting bacteria are able to attack the butter-fat directly. Secondly, proteolytic bacteria may break down the curd of the butter and so adversely affect the flavour. Many bacteria are both lipolytic and proteolytic. Some of these types produce undesirable changes more quickly than others, but even the slow-acting types may cause defects during long periods of transit and commercial storage.

Reasons have been given why butter made under good conditions should have a low total count including organisms of the above groups. Consequently, methods of testing which will be reasonably accurate when dealing with low counts are necessary. The accuracy of high counts is of less importance because it can be said at once that butter containing large numbers of bacteria must have been made from cream which has been seriously recontaminated after pasteurization; alternatively, it may have been stored under conditions which

facilitated the multiplication of the bacteria present, especially in unsalted butter. To deal with large numbers of samples simple and rapid tests are required. For the counting of yeasts and moulds in butter the microplate method devised by Johns (3) fulfils these conditions. Similarly, Barkworth (4) has shown that the Frost Little Plate method, as modified by Van Oijen, is particularly suitable for the routine testing of low-count clean milk. No difficulty was found in applying these methods to butter. By staining the cultures as recommended the results were available within 24 hr. This advantage was soon found to be outweighed by others when the cultures were incubated for several days. Counting could then be done easily without staining, by means of a 16× lens instead of a microscope, while characteristics developed which enabled some of the colonies to be classified. Thus the count could be made of lipolytic colonies, and of the so-called "heat-resistant" or yellow colonies. A preliminary account of the method, full details of which are now given, was published in 1937 (13).

Media

The nutrient agar now used for these counts contains the following: beef extract, 3 g.; salt, 5 g.; tryptone (Difco), 10 g.; agar, 18 g.; water, 1 l.; final *p*H 6·8. The absence of sugar should be noticed, the purpose being to discourage the growth of lactic streptococci. Although this type is probably present as a contaminant in most butters, a number of New Zealand factory managers make a practice of adding a small percentage of starter to their butter. Consequently it would be unfair to count starter bacteria as contaminants. In the earlier stages of the work Difco peptone was used instead of tryptone, but a comparison of the results of counts given by 130 samples showed that the tryptone medium gave distinctly higher figures in fully 50% of cases, as summarized in Table I.

Table I

	Total count	Heat-resistant count	Lipolytic count
Tryptone greater than peptone	67	68	63
Tryptone equal to peptone (±10%)	21	21	41
Tryptone less than peptone	42	35	26

For yeasts and moulds a synthetic medium devised by Davis (5) has been used, but in order to give more vigorous growth an increased quantity of dextrose and marmite has been found desirable. To allow for the rise in *p*H on mixing this medium with an equal portion of butter dilution two drops of glacial acetic acid should be added to each 10 ml. tube of medium, but too much acid may check the growth of yeasts. When made up according to the following formula this medium has been found preferable to a commercially prepared malt agar and whey agar for yeast counts.

A. Water	700 ml.
Ammonium nitrate $(NH_4)_3NO_3$	1 g.
Ammonium sulphate $(NH_4)SO_4$	1 g.
Dipotassium hydrogen phosphate K_2HPO_4	4 g.
Potassium dihydrogen phosphate KH_2PO_4	2 g.
Sodium chloride NaCl	1 g.
Dextrose	5 g.
Marmite	5 g.
Agar	20 g.

B. Water	300 ml
Citric acid (monohydrate)	5·7 g.

Sterilize A and B in separate flasks, mix and tube under sterile conditions; *p*H should be 3·5.

Sampling

A plug of butter is drawn with a trier which is clean but not necessarily sterile. If weighing is not done immediately the plug may be wrapped in a piece of clean butter paper, or placed in a sterilized bottle for transit to the laboratory. For weighing 1 g. portions a small knife or metal spatula is necessary. This may be conveniently sterilized (after wiping dry and clean) by dipping in methylated spirit; after draining a few moments the remaining spirit is ignited. After using the knife to remove the exterior of the plug, wiping and reflaming may be necessary before cutting off the 1 g. portion to be weighed into a sterile tube. These operations must be carried out without undue delay, although a few seconds may be allowed for the knife to cool after flaming. If the plugs of butter are hardened by cooling to 0° C. the portions can be weighed more easily. The butters are usually sampled within a few days after churning, in most cases before they are completely frozen. When necessary, the tubes with the samples are chilled prior to sending to the laboratory; or, for long distances, sent in an ice-box.

Culturing

A level warm table maintained at a temperature of about 45° C. is necessary. This may be conveniently arranged by means of a thermostatically controlled copper water-bath with a flat top. The water temperature may need to be rather higher to maintain the desired temperature on the top.

One tube each of nutrient agar and yeast-salt agar are melted, and a sterile pipette inserted into each through the cotton-wool plug, prior to placing in conveniently situated holes in the warm table. To replace the cotton-wool plugs we have found that rubber stopcaps are preferable (Baird and Tatlock, Ltd., Catalogue No. SD 4158). Each cap, bored to hold the pipette, can be sterilized with methylated spirits just before use. A tube with 10 ml. of medium suffices for about fifty samples, but to avoid delay in case of accidents it is useful to have ready melted another tube of each medium.

For each sample two 3×1 in. microscope slides are used, and both are marked with a rectangle 4×2 cm., by means of a yellow grease pencil. The slides for a large batch of samples may be prepared beforehand, and each sterilized by passing through the flame just before use. The two slides are placed, marked side up, on the warm table, one is labelled "A" and the other "YS", together with the sample number. The lid of a Petri dish serves to protect them from contamination.

To each tube with 1 g. of butter 9 ml. of sterile normal saline is added, preferably from a saline dispensing outfit.[1] The tube with butter and saline is melted at a temperature of 40–45° C. The lower temperature is preferable and care should be taken not to exceed the higher. Wood & Thornton (6) have shown that no variation in the bacterial content of the samples occurs when they are kept at a temperature of 43° C. for periods up to 4 hr. Nevertheless, we find that to have the best distribution of the fat globules in the dilution tube it is desirable to melt the butter just before plating.

The diluted butter sample is shaken for at least 30 sec. A sterile 1 ml. pipette is then used without delay to suck the mixture up and down vigorously several times prior to transferring 0·2 ml. to each of the slides, care being taken that the liquid is within the rectangle. These operations must be carried out as rapidly as possible in order to deposit on the slide an even mixture of saline and finely divided fat globules. To the slide marked "A" is added 0·2 ml. of agar and to that marked "YS" 0·2 ml. of yeast agar. The butter dilution and agar are mixed thoroughly with a sterilized platinum needle and spread evenly over the area. For speed and convenience it has been found satisfactory, with pipettes that deliver approximately 25 drops per ml., to use 5 drops of the butter dilution with 5 drops of the media. It is also

[1] We are indebted to a private communication (B.S.I. Draft of Standard Methods for Butter Examination), suggesting that the saline should be made up with 0·15% agar and we have found that this facilitates the emulsifying of the butter.

satisfactory to sterilize the platinum needle, mix first the yeast-agar culture, and then, without reflaming the needle, mix the tryptone-agar culture. The grease pencil marks are essential to prevent the agar spreading beyond the desired limits. The slides after mixing are promptly transferred to a piece of level plate glass and covered with a Petri dish while the agar hardens.

With the 1/10 dilution of the butter counts up to 200,000 per gram may be reasonably accurately estimated, but owing to the higher figures often yielded by unsalted butter an additional dilution of 1/100 in warm saline for the agar slides is desirable, as the 1/10 slides may be too crowded, especially for counting the lipolytic and heat-resistant colonies. In this case, for the lipolytic colonies (see later), 1 ml. of sterile butter-fat is added to a tube containing about 10 ml. of nutrient agar, which is shaken vigorously and used instead of the plain agar for the 1/100 culture. For estimating the coliform content 1 ml. of the 1/10 dilution is added to a tube of litmus milk which is incubated at 30° C. The amount of gas produced in this tube after 2–4 days is noted.

Incubating

The agar slides are incubated for at least 4 days at 30–32° C., while the yeast slides are kept at room temperature, about 20–22° C. Micro-cultures are usually incubated in a special moist chamber, but mould development may spoil many slides. Instead, we have found Petri dishes convenient, the bottom of each dish being covered with a circle of coarse filter-paper. After sterilizing, the paper is damped with a slight excess of freshly boiled water and after standing the surplus is poured off. Four-inch Petri dishes will hold two and 6-in. dishes six slides. Especially at the higher temperatures the interior of the incubator must be kept sufficiently moist, otherwise the colonies may not grow adequately. On the other hand, if the incubator is too moist, spreading types of colonies, which spoil the slides, are encouraged.

Counting

The rectangle covered by the media may be divided into eight or more equal parts by marking with a grease pencil on the underside of the slide. For large numbers of slides, however, it is more convenient to use a piece of plate glass about $2\frac{1}{2} \times 6$ in. on which the rectangle has been marked and subdivided with a diamond. The slide can then be placed on top of the marked portion and moved easily underneath the lens. To count the colonies a suitably mounted 16× lens is necessary. The slide, well illuminated from above, is placed on a flat black background. Since 0·2 ml. of the butter dilution is placed upon the slide the total number of colonies must be multiplied by 50 to give the count per gram of butter. When the count is very large a fraction of the slide is counted and a higher factor used. Early in this work a third slide was prepared, using agar from a tube to which had been added 1 ml. of butter-fat stained with Nile blue sulphate, per 10 ml. of agar. It was soon noticed that the lipolytic colonies could be observed on the ordinary agar slide, where they were surrounded and often covered by a circle or halo of white fat globules (as compared with the normal, transparent, glistening globules). The white appearance is no doubt due to hydrolysis to fatty acids, which, with their higher melting point, readily "crystallize" and develop the white halo. The adjoining photograph shows colonies (white) of different sizes and shapes, a few being surrounded by the halo of white fat globules; scattered about the background are many unchanged fat globules which vary in size, but are uniformly circular in outline, with a bright spot on one side. Magnification about 12×.

The yellow colonies (so-called "heat-resistant" types), can usually be easily counted after 4 days' incubation. Occasionally, however, they may be slow in developing the colour, so that more experience is required in counting them.

Further notes

When a new technique is proposed, comparisons are necessary with previously accepted methods. The procedure of procuring at random a 1 g. sample from a box of butter involves the use of much less material than has often been recommended. To check the reliability of the method duplicate samples were procured on various occasions from over fifty different churnings of butter. In more than half this number the duplicate sample was taken from a second plug drawn from a different part of the box. In the remaining cases the duplicates were weighed in sterile fashion from the same plug. In a few cases four 1 g. samples were taken from the same plug. The results of these duplicate tests, with a few exceptions, gave very satisfactory agreement (Table II). Arup & Gilmour(7) have noted that the very uneven distribution of bacteria in some butters may occasionally give rise to counts "clearly not representative of the whole box", probably due to faulty manufacture. The discrepancies observed may nevertheless quite well have been due to other errors of the method. There seemed no necessity to adopt a more complicated sampling procedure, or a larger sample, to eliminate the few cases where the agreement was not satisfactory. Further evidence of the reliability of the sampling method was obtained when tests (to be described later) were carried out upon surface scrapings.

For comparison, over eighty samples were cultured in duplicate on slides and in Petri plates, the 1/100 dilution being used for the latter. The counts on the microplates agreed very well with those on the Petri plates. On a few occasions some appreciable variations were observed, but these were really of little significance, as both counts were usually of the same order of magnitude. This is demonstrated by the scatter diagram (Text-fig. 1) based upon the logarithms of the respective figures. The fall of the points indicates the Petri plate figures, as a rule, to be somewhat higher. This is probably due to the colonies being less crowded when the 1/100 dilution is used upon a larger area of medium. In making such comparisons the limitations of the Petri plate itself must not be overlooked.

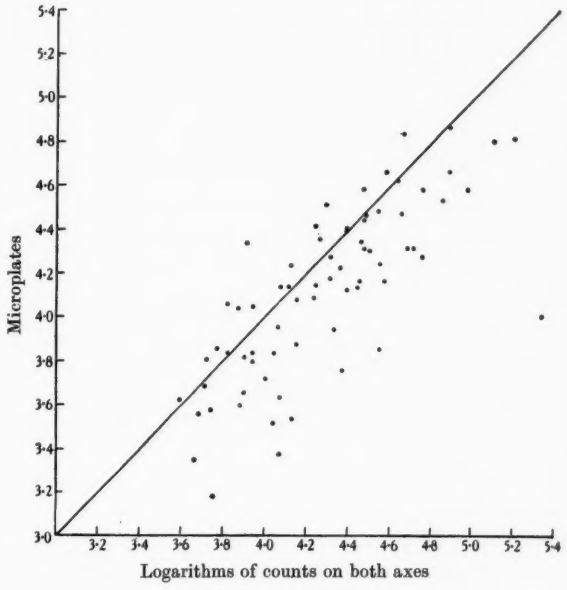
In comparing the counts of yellow or heat-resistant colonies by the two methods much less satisfactory agreement was obtained. This is due to the fact that the development of the yellow colour seems to be dependent upon a considerable number of factors, so that slight differences produced greater effects. A longer time of incubation yields more definite pigment, but this may be inconvenient especially if it involves a second handling of the microplates. In many cases the heat-resistant count amounted to 50% or more of the total agar count, and therefore no further efforts were made to obtain more precise comparisons of the heat-resistant count by the two methods. Heavily contaminated butter often gives cultures which are so crowded that the yellow colour does not develop. The heat-resistant count cannot then be made, but it may be assumed to be large.

Table II. *Results of tests upon duplicate samples of butter*

Sample no.	Churning no. and date	Grade and remarks	Bacteriological counts (per gram of butter) Class (see Table IV)	Total agar count	Heat-resistant organisms	Lipolytic organisms	Yeast count	Coliform organisms in 1/10 g. vol. of gas evolved
1	2/15	Finest	*aB*	4,600	2,000	50	150	Absent
1A	2/15	Duplicate	*B*	10,000	4,200	250	100	Absent
2	2/15	Finest	*B*	12,200	7,000	300	50	Absent
2A	2/15	Duplicate	*Ab*	8,400	4,400	50	100	Absent
3	1/16	First	*DD*	196,800	?	700	850	Absent
3A	1/16	Duplicate	*DD*	120,800	?	1250	850	Absent
4	1/16	First	*AA*	4,600	2,600	50	Under 50	Absent
4A	1/16	Duplicate	*AA*	2,600	1,400	Under 50	Under 50	Absent
5	1/15	First	*B*	30,000	16,400	Under 50	Under 50	Absent
5A	1/15	Duplicate	*B*	29,600	10,000	Under 50	Under 50	Absent
6	1/15	Finest	*B*	18,200	6,200	100	350	Absent
6A	1/15	Duplicate	*Ab*	9,400	3,600	Under 50	400	Absent
7	1/15	Finest	*BC*	28,800	21,600	100	350	Absent
7A	1/15	Duplicate	*bC*	56,000	21,600	100	150	Absent
8	2/17	Finest	*aB*	22,400	10,000	Under 50	50	Absent
8A	2/17	Duplicate	*Ab*	7,800	5,400	50	50	Absent
9	1/16	First	*C*	74,400	23,200	400	50	Absent
9A	1/16	Duplicate	*Bc*	34,800	19,200	100	100	Absent
10	4/15	First	*B*	18,000	11,600	150	150	Absent
10A	4/15	Duplicate	*A*	7,000	4,200	Under 50	100	Absent
11	2/16	Finest	*AB*	8,400	5,200	50	100	Trace
11A	2/16	Duplicate	*Bc*	10,000	7,000	50	Under 50	Much present
12	2/16	Finest	*B*	38,800	21,200	Under 50	Under 50	Absent
12A	2/16	Duplicate	*B*	40,000	20,800	Under 50	Under 50	Absent

The duplicate 1 g. portions were taken from a second plug of butter in each case.

Comparisons of the yeast counts by the two methods gave excellent agreement, and only in rare cases was any marked difference observed. Moulds were almost entirely absent. It had been expected that many samples would show mould contamination, and when very few were observed, even in several dozen samples of unsalted butter, we examined samples from the surface of the blocks of butter as well as from the interior. To obtain these scrapings the parchment was turned back from one side of the box and a sterilized metal edge used to scrape approximately 1 g. from an area of 15 sq. in. The whole scraping from this area was usually cultured, even though it was not exactly 1 g. If necessary, the counts could be recalculated on the basis of 1 g., but



Text-fig. 1. Petri plates.

instead of doing this it was considered preferable to keep to a uniform area. Out of a total of 134 which were examined during a certain period only eight gave a count of moulds, and of these only one had a high count. More than half the comparisons were incubated at 20° C., the remainder at 30–32° C. The other figures from these surface samples in most cases confirmed the counts of interior samples.

As previously mentioned, the lipolytic bacteria were at first counted by culture upon a medium containing fat stained with Nile blue sulphate. However, when this medium was used for both microplates and Petri plates the lipolytic counts agreed very well, but the total count in both cases was noticeably lower than that recorded in the other cultures. Other workers have commented upon the fact that this dye has a definite bactericidal effect. After

the improved method was devised whereby a lipolytic count could be made from the same microplate used for the total count, further comparisons were made. These showed that the figures obtained from microplates with Nile blue were distinctly too low. To confirm the lipolytic activity of an organism, we have frequently used the simple method of Berry (8), whereby a few streaks from a colony are made on a Petri plate containing butter-fat emulsified in the agar. After growth has occurred the plate is flooded with a strong solution of copper sulphate for 10 min. On pouring off and rinsing with clean water the fat globules which have been hydrolysed are found to be changed into the corresponding blue or green copper salts. Lipolytic colonies which occasionally grow on the yeast cultures seem to be a type of *Monilia*.

The use of agar containing tributyrin is now often recommended for counting lipolytic bacteria. Some forty-eight were cultured on Petri plates with this medium, but the results were not as satisfactory as with the microplate method described, although the total count of these Petri plates in most cases agreed very well with the microplate totals. Although Stark & Scheib (9) have found that certain types of bacteria (e.g. *Escherichia*) can hydrolyse tributyrin but not butter-fat, we have on occasion obtained lower counts of lipolytic bacteria with tributyrin agar than with the microplate method.

Other comparisons which were carried out involved the duplicate plating from the same dilution tubes by different workers. Counting was also carried out by another worker upon several batches. Both these operations, but especially the culturing, involve an appreciable amount of skill, so that one who is new to the technique cannot expect to get as consistent results as an experienced worker. The results of these comparative tests were in the main satisfactory.

The method used for detecting coliform contamination is that suggested by Grimes (10), and little work has been done to check its reliability. In the early stages of the work two litmus milk tubes were inoculated with 1 ml. from each butter dilution, and, as Grimes recommends, one tube was incubated at 37° C. and the other at 20° C. Since the object of using the lower temperature was to discover samples containing *aerogenes* types which do not develop at 37° C., it seemed probable that by incubating one tube at 30° C. both *coli* and *aerogenes* types would develop. Comparisons were therefore carried out upon 152 samples (Table III). On the basis of this work it was decided to substitute one tube incubated at 30° C. for the two tubes incubated at different temperatures.

During the early stages of the work all the culture slides were incubated at 20–22° C. After 4 days at this temperature satisfactory results were obtainable for total counts as well as for yeasts and moulds. Longer incubation scarcely altered these counts, but another 2 or 3 days were required to develop the pigment of the yellow colonies. As various workers have recommended a rather higher temperature, another incubator was procured to operate at 30–32° C. This has enabled the yellow colonies to be counted with the rest at 4 days, though in cases of doubt an extra day may still be allowed. Dr T. R.

Table III. *Coliform tests at different temperatures*

	Number of samples	
	30° C.	20° C.
Gas absent	89	101
Trace of gas	14	13
Gas present	33	34
Much gas present	16	4

No. of samples classed more favourably at 30° C. than 20° C. = 13.
No. of samples classed less favourably at 30° C. than 20° C. = 38.
No. of samples classed equally favourably at 30° C. and 20° C. = 101.
No of samples showing gas at 30° C. and not at 20° C. = 23.
No. of samples showing gas at 20° C. and not at 30° C. = 9.*

* In most cases only slight gas.

Vernon recently pointed out to us that the higher temperature is likely to check the growth of moulds upon the yeast cultures, so for these we have now reverted to the lower temperature, without detriment to the growth of yeasts. Since this temperature has been used, a tendency has been observed for small-mould counts to be recorded in a slightly higher percentage of cases.

Practical results

To communicate the results to the Dairy Division's Instructors and the factory managers concerned, the classification set out in Table IV is now used. The table at first used permitted higher numbers in the various groups, but experience indicated the feasibility of raising the standard. One important reason for using such a method of classifying the results is that when bacterial count figures are given to those who are unacquainted with the limitations of the methods which yield the figures, undue reliance may be placed upon small differences which may be quite within experimental error. Thus a broad scheme of classification can smooth out the errors of the various methods. For assessing the group letter from the results given by any particular sample which has yielded high figures by one test and low by another, it is often convenient to

Table IV. *Classification scheme*

Class	Interpretation	Total agar count	Count of heat-resistant organisms	Count of lipolytic organisms	Yeast count	Mould count	Coliform organisms in 1/10 g.
A	Good	Under 10,000	Under 5000	Under 50	Under 50	Absent	Absent
B	Satisfactory	10,000–25,000	5000–12,000	50–100	50–100	10–40	Trace
C	Poor	25,000–50,000	12,000–25,000	150–500	150–500	50–100	Present
D	Bad	Over 50,000	Over 25,000	Over 500	Over 500	Over 100	Much present

use two adjoining letters; e.g. *BC* indicates that the bacterial results classify the butter as intermediate between *B* and *C* group. If desired this may, however, be modified by writing one of the letters as a capital and the other in ordinary type, e.g. *bC* indicates that the results place the sample at the *B* end of the *C* group. Very bad samples may be marked *DD*, while samples which are *A* throughout are usually classified *AA*. With improved methods of cleansing the most difficult pieces of factory apparatus and regular attention to

detail it is hoped that the majority of factories may eventually attain regularly to the *A* standard.

Since this work has been in progress the results, which are regularly supplied to the Instructors, have frequently enabled them to search for insanitary conditions occurring in the factories, and thereby eliminate trouble before it has become sufficiently serious to affect the quality and grading of the butter.

The following examples of comments from instructors' reports may be cited.

The bacteriological report indicated a very high yeast count on O company's butter which for some time has been grading very well, finest, 94 points. As we look upon this as one of the well-kept plants, I considered it advisable to check up, and was much surprised to find ample justification for the report. An air chamber in the form of a 3 in. pipe about 18 in. long had been put in the churn-filling cream pipe to assist the pump. Although the Manager had instructed that this receive the same attention as the pipe itself this was not done, and it may be left to the imagination to think what it was like.

Bacteriological tests of this company's butter are anything but good, and I paid a visit to investigate the matter. It was found that the churnings, three in all, referred to in the last two reports came out of the same churn. On examining this churn a very bad fault was found behind one set of rollers at the end where the hole has been cut in the barrel for taking the roller out and in. It gave off a very foul odour and no doubt contributed to the high count of this butter.

With reference to the bacteriological examination that you have made of T company's butter, and the high counts obtained on various occasions, I thought that you would be interested to know that I dismantled the cream cooling plant to-day and found some of the cream pipes and two cream pumps unclean. I also found yellow slime on the inside surface of the top tray of one of the cream coolers. Needless to say, the plant received a thorough clean-up, and the necessary lecture on the sin of recontamination was given.

Visited C factory to ascertain if possible the cause of high counts in butter. Found churns in bad condition—wash water apparently not nearly hot enough. Arranged to demonstrate method with lime and boiling water. After rinsing churns, added water as hot as possible, approximately 170° F., with steam and water mixer; then made temporary connexion to churn with steam pipe, boiled water, added about four buckets clear lime water and half a bucket milk of lime. The churns were spun for $\frac{3}{4}$ hr. and drained, the water came out green and fatty. The manager and assistants expressed surprise at the much improved appearance of the churns when finished.

K factory manager concerned over high counts in butter. Creamery is well kept but churns not in good sanitary condition—water used for cleaning not hot enough. Churns limed out with boiling water and spun for 45 min. This procedure will be followed in future.

In addition to revealing factories requiring attention to improve their sanitary condition, the bacteriological counts provide the instructor with evidence which he can use in conjunction with his findings at the factory in order to convince the manager that extra attention is a necessity. Consequently the managers have become very interested in obtaining from the instructors these results as a check on the efficiency of the cleaning operations.

Experiments for improvements

In addition to the various trials and comparisons which were carried out during the early stages of developing these methods, opportunities have been taken to try out various alternative procedures for increasing the amount of useful information provided by the tests. The principal feature not covered by the above methods is the number of proteolytic bacteria present in any sample of butter. For this purpose various methods have been tried. The first attempts were based upon the use of milk agar such as is frequently used for culturing milk and other dairy products. The microplate total counts, with milk and nutrient agars, agreed fairly well with a slight tendency for milk agar figures to be lower. Milk agar Petri plates usually gave slightly higher total counts than the milk agar microplates, but agreed well with the nutrient agar Petri plates. Unfortunately proteolytic colonies did not show up on the microplates with milk agar unless the amount of milk was increased to 5%, probably owing to the thinness of the agar layer. One feature noticed with milk agar was the more definite development of colour by the yellow colonies. Several attempts have been made to use the casein agar of Frazier & Rupp (11) (made according to the modification of the National Institute for Research in Dairying) for counting proteolytic types on microplates. In contrast to its use with Petri plates the results were not very encouraging. Counts could be made at 24–48 hr., but the results were usually quite low as compared with Petri plate counts. When the microplates were incubated longer the zones had usually spread over the whole area so that no count could be made. With one batch on Petri plates there was a definite relationship between the count of proteolytic organisms and the heat-resistant counts, possibly due to the fact that many of the latter type are proteolytic micrococci. The ordinary strength of casein in this medium proved quite inadequate for microplates owing to the thinness of the layer of medium. More satisfactory zones were, however, obtained when the amounts of casein and solvent lime water were increased two-fold or three-fold.

Comparisons, upon Petri plates, were made of milk agar and gelatine cultures, but there seemed to be no correlation between caseolytic and gelatine liquefying types. For this and other reasons it proved unsatisfactory to inoculate tubes of gelatine with 1 ml. of the butter dilution.

The most satisfactory method of ascertaining the proteolytic count, without utilizing Petri plates, was found to be by means of the Esmarch Roll Tube method using agar with triple strength casein and putting 1 ml. of the 1/10 dilution into 2 ml. of the medium in the tube. To follow this procedure, owing to the nature of the medium, involves much extra trouble which may well be unwarranted. The methods we have devised provide a very good indication of the sanitary conditions in a butter factory; so although a method to ascertain the proteolytic count in butter may be useful in certain cases, we feel doubtful of the need for adding it to what is already a very satisfactory and expeditious

routine method, especially as Jacobsen (12) and others have shown that the majority of lipolytic bacteria are also proteolytic. In cases where a butter sample is definitely suspected to be of inferior quality, due to bacterial growth, a count of proteolytic bacteria is certainly desirable and for that purpose the Petri plate method may be employed until some simpler alternative is devised.

To ascertain more precisely the coliform content of butter samples, a number of trials have been carried out with culture media, such as "Difco" violet-red-bile-agar, which would give actual counts. In some cases the microplate results seemed to be satisfactory, while in others the typical colonies did not develop when expected. One reason for this was thought to be the age of the medium which appears not to give good results unless freshly made up. Other similar trials are contemplated.

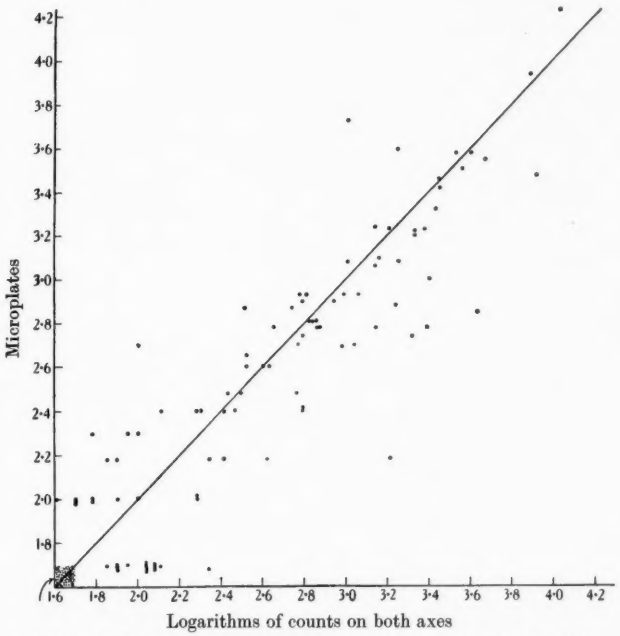
Since the counts are all obtained by multiplying the culture figures by 50, types present in numbers less than this figure cannot be accurately enumerated. This is of most importance in connexion with moulds which, under the best conditions, should be entirely absent from the butter. To check the mould results, comparisons of microplate and Petri plate figures have been made upon seventy-two samples. These reveal that a small proportion of mould counts between ten and fifty are not detected, but the majority of counts of fifty or above are revealed by the microplate method.

With the use of a lower incubating temperature for the yeast cultures there has been a tendency for higher mould figures to be obtained. Some attention has therefore recently been given to the roll-tube method to replace the microplate used for the yeast and mould count. Encouraging results have been obtained by inoculating 1 ml. of the 1/10 dilution into a tube (6″ or 7″ × ¾″) containing 2 ml. of the melted yeast medium. After shaking to mix, the tube is held almost horizontal and rotated rapidly to distribute the medium to within an inch of the plug. Meanwhile the tube is held under a cold tap to solidify the agar, after which it is incubated plug downwards. More satisfactory results are obtained when the percentage of agar in the medium is increased from two to three, but no acetic acid need be added as for the microplates. With this method care must be taken to ensure that the medium is not too hot. The tubes may be kept melted in a bath of hot water and transferred to a cooler bath at 42–45° C. a few minutes before use. After inoculation, the tubes must be rolled without undue delay, but they may be left in the water bath until the total count microplate is disposed of. With experience the roll-tube can be completed almost, if not quite, as quickly as the microplate culture.

In further comparisons, using the roll-tube and the microplate at 20–22° C. for each of 172 samples, very satisfactory agreement of the yeast counts has been obtained (Text-fig. 2). Of these samples, ninety-one were also examined upon Petri plates, the yeast counts agreeing well with those given by the other methods. Text-fig. 3 demonstrates the agreement between the Petri plates and the roll-tube figures. The latter (like the microplate results shown in Text-fig. 1) tend to be slightly lower, probably due to crowding. In Table V are summarized

Table V. *Comparison of mould-count methods*

Results by roll-tubes	Results by microplates			
	Under 50	50–100	150–500	Totals
Under 10	114	6	—	120
10–50	33	13	0	46
60–100	6	3	1	10
110–500	—	3	1	4
Totals	153	25	2	180

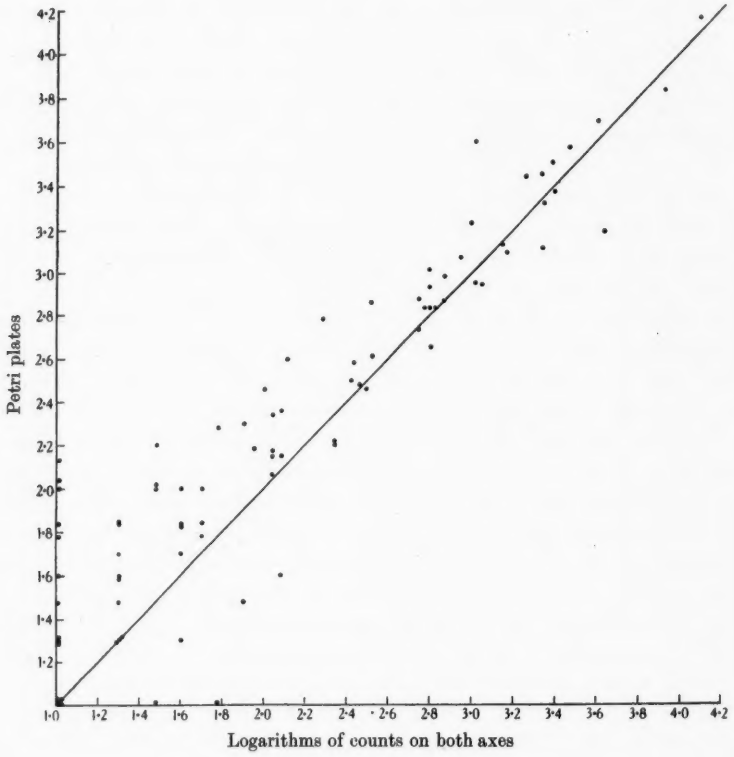


Text-fig. 2. Roll tubes. In the left-hand corner are 79 pairs which in both cases gave counts of 50 or less

the mould results of 179 samples tested by both roll-tubes and microplates. (About a third of the samples were surface scrapings.) Although the agreement is satisfactory for a large proportion of the comparisons, the larger inoculum used with the roll-tubes seems likely to be more reliable, especially in dealing with low figures, because of the irregular distribution of the moulds. Apart from the fact that the factor 10 instead of 50 may be used, the roll-tube has the important advantage that it is less subject to chance contamination than the micro- or the Petri plate. The foregoing comparisons have encouraged us to adopt the roll-tube instead of the microplate for yeast and mould counts.

The low mould counts revealed by these tests agree with the results of similar routine tests by Irish (7) and Canadian (6) workers. The question therefore arises as to the origin of the occasional complaints of spoilage of butter by mould growth. Interior mould development within the block—fortunately a very rare occurrence—is usually due to growth of the *Cladosporium* type.

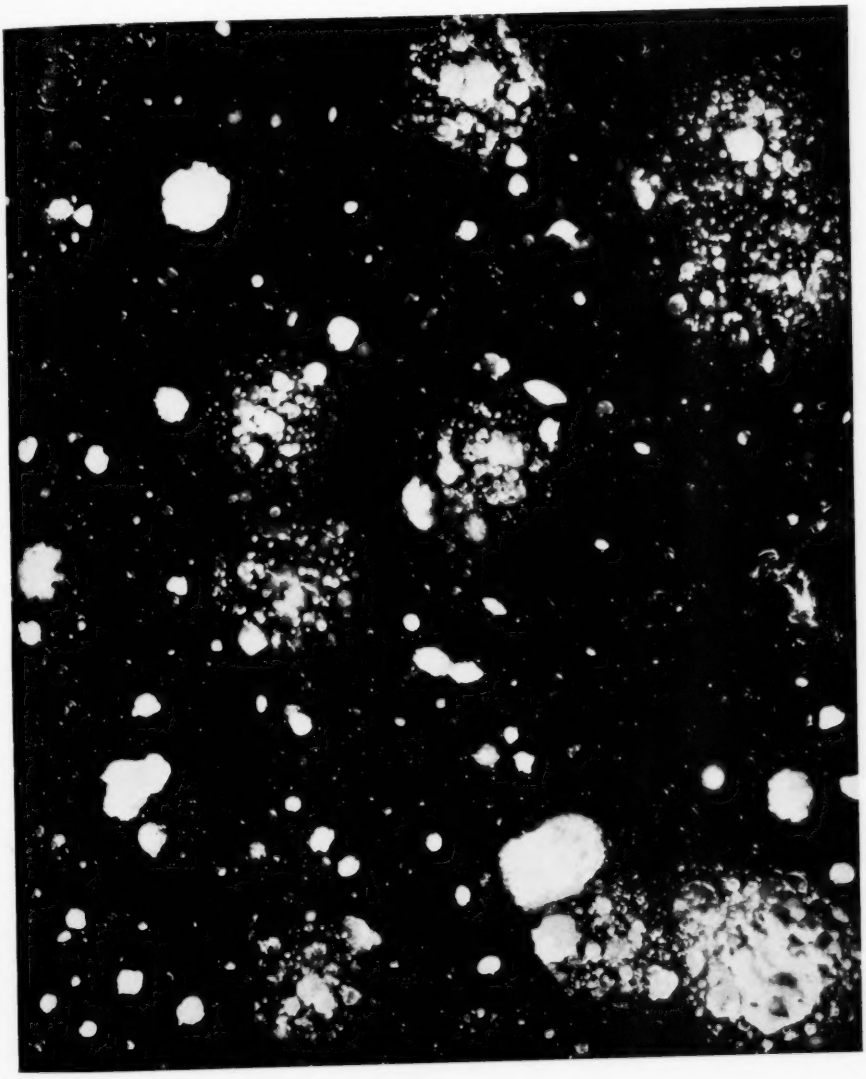
Copious mould growth over the exterior of the block of butter is more frequently observed. Since so many surface scrapings procured shortly after manufacture have also yielded low mould figures, one must conclude that the growth of exterior mould is closely related to external storage conditions, particularly those leading to condensation of moisture, a fact emphasized by various



Text-fig. 3. Roll tubes.

workers. Inferior storage conditions may occasionally stimulate mould growth without this reaching the visible stage; therefore the examination of surface scrapings taken after transit and storage in England, may provide a useful means of detecting and eliminating faulty conditions before they have persisted long enough to give rise to complaints. Arup & Gilmour (7) have shown that at ordinary temperatures the yeast count of butter may increase enormously. Consequently if butter is found to have a high yeast count on the surface while the interior figure is low it may perhaps have undergone a period of unsatisfactory storage.

Notwithstanding continual efforts to ensure the transit and storage of butter under the best conditions, unforeseen circumstances may occasionally



MOIR AND RUSSELL—BACTERIOLOGICAL TESTING OF BUTTER

give rise to adverse conditions. Any organisms present within or on the surface of the butter may then multiply to numbers which may be harmful, especially in unsalted butter. The insistence upon the highest possible standard of factory sanitation should permit butter to be produced with a minimum bacterial content. Small original numbers of organisms are less likely to increase sufficiently to give rise to deterioration under adverse storage conditions. This may be of especial value in maintaining the quality of the butter after defrosting, while on its way to the consumer.

Summary

Simple and rapid methods have been devised which utilize the minimum of apparatus and material for the routine bacteriological testing of butter. These involve the preparation of microplate cultures to obtain counts of total bacteria, "heat-resistant" bacteria, lipolytic organisms, yeasts, moulds; Grimes's method as a measure of the coliform content is also used.

The method for counting colonies of lipolytic bacteria is based upon their ability to produce round each colony a halo of white solid fatty acids in place of the clear glistening fat globules.

The new methods have been exhaustively compared with those hitherto accepted, and have been found to give satisfactory and reliable results.

A variety of comparisons has been carried out to improve still further the methods in use and to provide additional information about the nature of the bacteria contamination present in the butter. The roll-tube method seems preferable to the microplate for mould counts, and is quite suitable for yeast counts.

The results obtained confirm the view expressed by Wood & Thornton (6) that yeast and mould counts provide an inadequate criterion of factory sanitation, and should be supported at least by the total count of bacteria.

The employment of these methods has enabled large numbers of samples to be dealt with. Thus samples from most of the principal New Zealand butter factories have been regularly tested. The information so obtained has been of material value to the Dairy Division's Instructors in drawing attention to factories where sanitary conditions left room for improvement.

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230. THE RELATION OF CERTAIN LACTIC ACID BACTERIA TO OPEN TEXTURE IN CHEDDAR CHEESE

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THE three types of open texture which occur in New Zealand Cheddar cheese were described in the *Annual Report* of this Institute for 1929–30(1). The type there referred to as "slit" openness (the name is sufficiently descriptive of its character) is the most widespread; it is the most troublesome defect in Cheddar cheese.

When work was initiated here on slit openness there was a natural tendency to consider that the defect might be caused by the production of gas in the cheese by bacteria, in spite of the fact that the slits were so different in appearance from the gas holes ("eyes") which occur in Emmenthal cheese(2) and sometimes in "Sweet" Cheddar cheese.[1] It was soon found, however, that the occurrence of slit openness was not correlated directly with the presence in cheese milk of large numbers of any of the commoner gas-producing contaminant bacteria. Milk of low bacterial count and pasteurized milk frequently gave cheese in which slit openness developed. In view of these facts the bacteriological aspect of the subject was abandoned temporarily and an extensive trial was carried out of the various remedies for texture defects which are suggested in text-books on cheese manufacture. At the same time the effects of many modifications on generally accepted New Zealand cheese-making practice were investigated. In the course of this work it was found that certain variations in procedure tended to reduce the incidence of openness, but it was quite evident in the course of many manufacturing experiments(4) that the fundamental cause of slit formation had not been discovered.

Experience over the course of several years gradually led to a renewed attack from the bacteriological side. Prof. W. Riddet pointed out that much of the evidence accumulated incidentally during cheese-making experiments suggested that some of the normal flora either in starter cultures or in ripening cheese of good flavour might be implicated in the formation of slits. In other words, it was necessary to consider as potential sources of trouble some of the true lactic acid bacteria rather than the previously suspected contaminants like the coliform bacilli.

[1] After the major part of this investigation had been completed the recent paper of van Beynum & Pette(3) on openness in Edam cheese appeared. These investigators demonstrated that excessive openness in that type of cheese, presenting an appearance similar to fermentation holes in Cheddar cheese, is caused by the formation of gas by *Cl. tyrobutyricum*.

In the present work the problem of slit openness has been re-examined from the bacteriological aspect and the following points have been established:

I. "Open" cheese evolve carbon dioxide more rapidly than do "close" cheese of the same age.

II. "Open" cheese usually contain a larger number of rapid gas-forming strains of lactic acid bacteria than do "close" cheese.

III. The inoculation of cheese-milk with these comparatively rapid gas-producing strains of lactic acid bacteria leads to the development of slit openness in cheese.

I. THE RELATIVE RATES OF EVOLUTION OF CARBON DIOXIDE BY CLOSE AND BY OPEN CHEESE

The fact that carbon dioxide is formed in Cheddar cheese during the process of ripening is well known(5). In order to decide whether there was any possibility of slit openness arising from the comparatively rapid formation of carbon dioxide within the cheese, the relative amounts of this gas given off in a fixed time by approximately fifty "close" and fifty "open" cheese were determined. (The "closeness" or "openness" of these cheese had been previously recorded by experienced graders.) The cheese were arranged in pairs of approximately the same age, one cheese in each pair being close and the other open. The relative rates of evolution of carbon dioxide by the cheese were determined by a modification of the Eldredge method(6):

25 g. of each cheese, cut into small pieces under aseptic conditions, were placed in a sterile 100 ml. flask which was connected with a second 100 ml. flask containing $N/10$ barium hydroxide, by means of a sterile U-tube loosely

Table I. *The relative rates of evolution of carbon dioxide from close and from open cheese*

Cheese no.	Age (months)	Rate of evolution of carbon dioxide (ml. $N/10$ barium hydroxide solution neutralized in 2 days at room temperature)
1	6	1·7
2	6	3·4
3	6	0·6
4	6	3·5
5	5	1·4
6	5	7·1
7	3	2·1
8	3	3·5
9	3	2·0
10	3	8·0
11	3	2·2
12	3	7·1
13	2	1·1
14	2	6·5
15	2	1·7
16	2	5·6
17	1	1·9
18	1	4·6

Note. Odd numbers, close cheese; even numbers, open cheese.

plugged with cotton-wool. The amount of carbon dioxide which passed over in 2 days, at room temperature, was measured.

A representative series of results is given in Table I.

It will be seen that, in each pair of cheese, the open cheese evolved carbon dioxide more rapidly than did the close cheese.

This finding was confirmed, moreover, by examination of a large number of canned cheese obtained from various factories. It was found that the relative bulging of the containers gave a rough indication of the degree of slit openness present in the cheese, swollen tins usually containing open cheese while unswollen tins generally contained close cheese.

II. The relative rates of formation of carbon dioxide by different strains of lactic acid bacteria isolated from open cheese and from close cheese

The formation of carbon dioxide by different types of lactic acid bacteria has been studied by many workers (7-12). The development of "eyes" in Emmenthal cheese was attributed by Freudenreich and Orla-Jensen (2) to the formation of carbon dioxide by strains of propionic acid bacteria. Later, Orla-Jensen (13), in his monograph on the lactic acid bacteria, included gas formation as one of the distinguishing characteristics of the genera *Betacoccus* and *Betabacterium*.

In the present investigation the relative rates of formation of carbon dioxide by strains of lactic acid bacteria isolated from open cheese and from close cheese were compared. The method used was similar to that described in the preceding section: The strain under study was sown into 50 ml. autoclaved milk containing 5% of yeast extract in a 100 ml. Erlenmeyer flask and the carbon dioxide evolved in 6 days at 22° C. was absorbed in standard barium hydroxide solution.

In Table II are shown the relative rates of production of carbon dioxide by 10 strains of lactic acid bacteria picked off from adjacent colonies on plates (yeast-whey-agar) prepared from each of two typical close cheese and two typical open cheese.

It will be seen that the strains of streptococci and lactobacilli isolated from the two close cheese formed carbon dioxide relatively slowly in yeast milk. On the other hand, the flora of the two open cheese included strains of betacocci (cheese 2) and of lactobacilli (cheese 4) which produced carbon dioxide rapidly in yeast milk. The streptococci isolated from the immature cheese 1 and 2 were probably derived from the starter.

Although, in general, open cheese were found to contain lactobacilli or betacocci capable of producing carbon dioxide rapidly in yeast milk, yet from occasional open cheese, which themselves evolved large amounts of gas, only strains of lactic acid bacteria which formed carbon dioxide slowly in yeast milk could be isolated. It is possible, in such instances, that some of the

Table II. *The relative rates of formation of carbon dioxide by different strains of lactic acid bacteria isolated from two close cheese and from two open cheese*

Cheese examined			Organisms isolated		
Cheese no.	Age months	Whether close or open	Strain no.	Whether *Streptococcus* (*S*) *Betacoccus* (*B*) or *Lactobacillus* (*L*)	Amount of carbon dioxide evolved in 6 days at 22° C. (ml. *N*/10 $Ba(OH)_2$ neutralized
1	1	Close	1	*L*	1·6
			2	*L*	1·5
			3	*L*	1·2
			4	*L*	1·7
			5	*L*	1·3
			6	*L*	1·3
			7	*S*	1·3
			8	*L*	1·4
			9	*L*	1·6
			10	*S*	1·2
2	1	Open	1	*B*	20·1
			2	*S*	1·1
			3	*L*	3·6
			4	*S*	2·1
			5	*S*	3·2
			6	*B*	20·4
			7	*L*	3·4
			8	*B*	20·3
			9	*B*	20·5
			10	*B*	21·4
3	6	Close	1	*L*	3·4
			2	*L*	4·1
			3	*L*	4·1
			4	*L*	4·4
			5	*L*	4·6
			6	*L*	4·1
			7	*L*	4·3
			8	*L*	4·0
			9	*L*	4·0
			10	*L*	3·8
4	5	Open	1	*L*	2·0
			2	*L*	8·3
			3	*L*	1·2
			4	*L*	9·6
			5	*L*	2·1
			6	*L*	2·0
			7	*L*	17·3
			8	*L*	1·7
			9	*L*	8·6
			10	*L*	1·9

strains present in the cheese were capable of producing gas more rapidly in that medium than in yeast milk. Certain strains of lactobacilli are known to behave in this manner, e.g. strain no. 7 (Tables III and IV).

It should be mentioned that even from close cheese (especially when mature) it was frequently possible to isolate by an enrichment method (14) gas-producing strains of lactobacilli. Apparently, the presence of very small numbers of gas-producing strains does not cause slit openness. (This has been confirmed experimentally. See Table III.) Moreover, the initial presence of appreciable numbers of gas-producing organisms does not necessarily lead to the development of slit openness (4), since certain physical and chemical conditions in the cheese may control the growth of these organisms or modify the effects which they would normally produce.

III. The production of slit openness in Cheddar cheese by inoculation of the cheese milk with strains of lactic acid bacteria capable of producing carbon dioxide rapidly

Preparation of cultures. Cultures of the various organisms (mainly lactobacilli) were prepared in sterile milk containing 5% of yeast extract. After incubation at 30° C. until the milk clotted, the cultures were added to the cheese milk either with the starter or at the same time as the rennet.

Method of cheese manufacture. The method employed has been described in an earlier publication (14). Milk of good quality was pasteurized by the flash method (155°) or the batch method (143–145°/15 min.) and divided between two 80 gal. vats, one of which served for manufacture of control cheese, while the other, employed for manufacture of the inoculated cheese, was housed in a separate room and attended by an isolated cheese-maker. All practicable precautions were taken to avoid contamination of the control vat, and as nearly as possible identical manufacturing conditions were observed for both vats. From each vat one 40 lb. or 80 lb. cheese and one 10 lb. cheese were made; the 10 lb. cheese were canned and all the cheese were ripened in curing rooms at a temperature of 60° F. for 14 days and subsequently at 45–50° F. At an age of 3–4 months the cheese were cut in half and examined.

Results. In all, a wide variety of lactic acid bacteria was added to the cheese milk and a considerable number of cheese were manufactured. From examination of these cheese it was found that strains of lactobacilli (and betacocci) capable of producing carbon dioxide rapidly caused slit openness to develop in the cheese, whereas strains of lactobacilli which formed carbon dioxide relatively slowly had little or no effect on the cheese texture.

In Tables III and IV, 11 representative strains of lactobacilli and betacocci which were found to produce slit openness will be dealt with.

Table III. *The effect of gas-producing strains of lactobacilli (and betacocci) upon the texture of cheese*

+ + + Signifies that the inoculated cheese were considerably more open than the control cheese.
+ + Signifies that the inoculated cheese were definitely more open than the control cheese.
+ Signifies that the inoculated cheese were slightly more open than the control cheese.
− Signifies that the inoculated cheese were indistinguishable from the control cheese.

Strain	Percentage inoculum (clotted yeast-milk culture)	No. of cheese manufactured (pairs)	Extent of slit openness produced
1	0·001–0·2	23	+ + +
	0·0000001–0·000001	5	−
2	0·001–0·004	4	+ +
3	0·001–0·004	4	+ +
4	0·001–0·004	4	+
5	0·001–0·004	4	+
6	0·1–0·2	4	+
7	0·1–0·2	15	+ +
8	0·1–0·2	4	+
9	0·1–0·2	5	+ +
10	0·1–0·2	5	+ +
11	0·2	3	+ +

Strains 1–5 in Tables III and IV would appear to be betabacteria (13). These strains produced large amounts of carbon dioxide and some volatile acid, formed inactive lactic acid in yeast-milk, were greatly stimulated by yeast extract, produced a low final acidity in milk and failed to ferment starch, inulin, dextrin and salicin (strain 1 anomalous). Although strains 2–5 were isolated from cheese made in different localities, it is clear that they were very similar.

These five strains were probably *Betabacterium breve* (13), or *Lactobacillus brevis* (Orla-Jensen) Bergey *et al.*, according to Pederson (15), since they fermented arabinose, xylose, dextrose, laevulose, galactose and maltose, failed to ferment salicin (except strain 1) sorbitol, glycerol, rhamnose, dextrin, inulin and starch, formed short rods, approximately $0{\cdot}7–1{\cdot}0 \times 1{\cdot}0–4{\cdot}0\mu$ in milk and grew best at 30–35° C. (optimum temperature often slightly lower in yeast milk than in milk).

Strain 6 formed acid in dextrose, laevulose, mannose, galactose, sucrose and raffinose (but not in maltose) and failed to ferment arabinose, xylose, rhamnose, sorbitol, mannitol, inulin, dextrin, starch and salicin. Sugar reactions, therefore, tended to relate this organism to *Lactobacillus fermentum* Beijerinck (*Betabacterium longum* Orla-Jensen), and in agreement with this, the optimum, minimum and maximum temperatures were somewhat higher than those of strains 1–5. In most other respects, however, strain 6 resembled *Sbm. plantarum*.

Strain 7 was probably *Lactobacillus plantarum* (*Streptobacterium plantarum*), since it grew faster in milk than did strains 1–5, was stimulated by yeast extract (16,17), formed inactive lactic acid, produced in milk final *p*H's intermediate between those given by betabacteria and by *Sbm. casei* and gave typical sugar reactions. Strain 7 produced carbon dioxide fairly slowly in yeast milk, but relatively rapidly in cheese—probably on account of its high salt tolerance (Table IV) which would allow this strain to grow faster than most other strains in cheese.

Strain 8 would seem to be an intermediate form (16,17) between *Sbm. casei* and *Sbm. plantarum*. It resembled *casei* in rapidly reducing litmus milk, attacking casein, producing acid in milk faster than *plantarum* and developing a higher final acidity. The resemblance to *plantarum* was shown by the production of inactive acid and the fermentation of raffinose and inulin.

The coccal strains 9, 10 and 11, forming large quantities of gas and volatile acid and growing poorly in milk, were betacocci (*Leuconostoc*). Strains 9 and 10, which were isolated from cheese, were much more active than strain 11 which was isolated from a commercial starter culture, and were somewhat irregular in shape. The sugar reactions of these two strains agreed with *Leuconostoc dextranicus* (18). Strain 11 was probably *Leuconostoc citrovorus* (18), although it was slightly irregular in that it formed small amounts of acid in xylose, saccharose and raffinose.

Table IV. *Some biochemical characteristics of the eleven gas-producing strains of lactobacilli and betacocci*

(Unless otherwise indicated, the temperature of incubation employed was, in all cases, 30° C.) All were Gram-positive; they did not form spores, reduce nitrate, produce catalase or liquefy gelatine. Surface growth on agar media under aerobic conditions was poor and colonies were typically small.

Strain	Morphology after 2 days' growth		Relative rates of production of carbon dioxide in	
	(*a*) In milk	(*b*) On yeast whey agar	(*a*) Yeast milk (ml. *N*/10 barium hydroxide neutralized in 14 days at 22° C.)	(*b*) Yeast milk containing 5% salt
1	0·7–0·9 × 2–4 μ. Singly and very short chains	0·7–0·9 × 4–7 μ. Short chains	51·7	30·2
2	Similar to 1	Similar to 1, but rods slightly shorter	42·9	6·3
3	Similar to 1	Similar to 2	29·0	7·8
4	Similar to 1	Similar to 1	34·4	4·2
5	Similar to 1	0·8–1·0 × 4–7 μ. Short chains	28·6	5·4
6	0·4–0·6 × 2–6 μ. Singly and in short chains	0·8–1·0 × 2–7 μ, heavily stained. Short chains	26·3	5·3
7	0·6–0·9 × 3–8 μ. Short chains	0·8–1·1 × 3–5 μ. Short chains	11·2	9·1
8	0·4–0·7 × 2–8 μ. Somewhat granulated and poorly stained	0·6–0·8 × 3–5 μ. Short chains	23·3	4·5
9	Cocci 0·3–0·5 × 0·3–0·5 μ. Short chains	0·3–0·7 μ, poorly shaped, rod-like	81·1	20·2
10	Cocci 0·3–0·5 μ. Short chains containing occasional "swollen" units	0·5–0·8 μ. Short chains	77·2	17·2
11	Cocci 0·3–0·4 μ. Very short chains	Cocci 0·3–0·4 μ, poorly shaped	57·9	15·6

Strain	Reduction of litmus milk (no. of days)	Total acid formed in 14 days (expressed as g./l. lactic acid)		Final *p*H produced after 50 days' growth in		Type of lactic acid formed in yeast milk	Amount of volatile acid formed in 14 days (expressed as % total acid)	
		(*a*) Milk	(*b*) Yeast milk	(*a*) Milk	(*b*) Yeast milk		Yeast milk	Yeast milk + 0·4% citric acid
1	∞	2·07	6·84	4·35	3·79	l	9·3	10·4
2	∞	1·89	7·11	4·31	3·85	l	10·5	12·0
3	∞	1·80	6·84	4·33	3·85	l	11·7	11·1
4	∞	1·53	7·29	4·48	3·93	l	33·5	84·5
5	∞	0·81	1·44	4·67	4·01	l	60·8	93·0
6	∞	3·78	9·09	4·02	3·76	l	12·8	13·1
7	∞	5·22	9·72	3·98	3·77	l	12·6	10·9
8	7	8·73	10·17	3·87	3·69	l	12·1	33·6
9	15 (weak reduction)	4·59	5·40	4·67	4·51	l	30·0	59·0
10	16 (weak reduction)	2·43	5·31	4·85	4·53	l	22·0	>90
11	∞	0·45	3·78	5·12	4·72	l	35·9	>90

Table IV (*continued*)

Sugar reactions. (Casein-digest broth, containing 0·5% nitrogen.) Acid formed in 14 days expressed as g./l. lactic acid. (– represents less than 0·10% lactic acid)

Strain	Approximate optimum temperature*		Thermal death-point (° C.) (15 min. exposure)	Arabinose	Xylose	Rhamnose	Mannose	Mannitol	Dextrose	Laevulose	Galactose	Sorbitol	Sacharose	Lactose	Maltose	Raffinose	Salicin	Dextrin	Inulin	Starch	Glycerol
	Milk	Yeast milk																			
1	30°	30	70	7·0	5·2	—	—	2·5	1·6	4·7	1·4	—	2·2	1·8	7·7	—	1·1	—	—	—	—
2	35	32	70	3·4	5·8	—	—	1·1	1·6	5·6	1·8	—	—	1·8	1·6	—	—	—	—	—	—
3	35	32	75	3·2	5·9	—	—	1·6	1·8	5·8	1·6	—	—	1·8	1·8	—	—	—	—	—	—
4	35	30	70	3·8	4·9	—	—	1·6	2·0	5·4	2·0	—	—	2·2	1·8	—	—	—	—	—	—
5	35	35	70	2·0	3·8	—	—	1·1	1·8	5·8	1·4	—	—	1·6	2·2	—	—	—	—	—	—
6	40	35	75	—	—	—	4·5	—	5·9	5·0	2·3	—	3·8	4·0	—	4·7	—	—	—	—	—
7	35	30	65	6·1	1·4	2·3	4·0	—	3·4	4·7	2·7	3·8	3·8	4·3	5·0	2·9	4·0	1·6	—	—	1·0
8	30	30	65	—	—	—	2·9	1·4	4·3	4·3	1·8	—	1·3	3·1	3·8	1·0	2·2	1·8	—	—	1·1
9	30	25	65	—	—	—	1·1	—	2·2	1·4	1·4	—	4·1	3·6	3·8	3·6	—	—	—	—	—
10	30	25	70	—	—	—	2·3	—	2·7	1·8	2·0	—	2·9	3·2	2·9	2·5	—	—	—	—	—
11	30	30	65	—	2·0	—	—	—	1·7	1·1	1·0	—	1·3	1·0	—	2·1	—	—	—	—	—

* Minimum and maximum temperatures have not been included in this table, since there was no significant variation (except in the case of strain no. 6 with minimum temp. 15°, maximum temp. 45°), minimum temperatures ranging from 10 to 12° C., and maximum temperatures from 40 to 44° C. The method of estimating volatile acidity has been described in an earlier publication(14).

Discussion

In the preceding sections evidence has been given in support of the view that slit openness in Cheddar cheese is associated with the production of gas within the cheese. The flora of open cheese has been shown to include a larger proportion of gas-producing types of lactic acid bacteria than does the flora of close cheese. Finally, the inoculation of cheese milk with these gas-producing strains has led to the development of slit-openness in the resulting cheese.

The types of lactic acid bacteria which most commonly appear to be responsible for the development of slit openness are the gas-producing lactobacilli (or betabacteria), especially *L. brevis* (Orla-Jensen) Bergey *et al.* Less frequently openness seems to result from the presence of certain strains of *Sbm. plantarum* and strains intermediate between *Sbm. plantarum* and *Sbm. casei*. Certain strains of betacocci have also been found to produce slit openness. In fact it appears probable that any lactic acid rod or coccus capable of fairly rapid gas production in cheese is liable to cause slit openness.

The immediate source of the causative organisms is probably, in most instances, the cheese milk. The bacterial content of the rennet used in New Zealand is very low and commercial starter cultures contain either few or no lactobacilli. Some commercial starters, however, contain strains of *Leuconostoc*, especially *Leuconostoc citrovorus*, and it is possible that, occasionally, the numbers of these organisms added to the cheese milk with the starter may be sufficiently high to cause a certain amount of slit openness to develop in the cheese.

The distribution of gas-producing organisms in cheese

In young cheese there was observed a general tendency for gas-producing organisms to be present in the vicinity of slits in greater numbers than in the unshattered parts of the cheese. It would seem that once slits have formed as a result of gas production by betabacteria or other types, the moist surfaces of the slits permit these organisms to spread over a large area. The further production of carbon dioxide along the slits enlarges the openings, and so the process continues. This is probably the reason why slits frequently extend for considerable distances in cheese (4). As the cheese ripens, the distribution of the various types of bacteria tends to become more uniform.

The relation of fermentation openness to slit openness

Some light was thrown on this point by examination of cheese made with the addition of strain 1 (Table III). Some of the cheese were "inclined sweet" (the *p*H at 14 days being 4·95 in place of the normal value 4·80–4·90) and these contained fermentation holes in addition to slits, both apparently having been produced through the formation of gas by strain 1. The acidity of the curd seemingly governs the development of fermentation openness.

While it would appear that the organisms commonly responsible for the production of slit openness in Cheddar cheese have been identified, the eradi-

cation of the defect is quite another question. A number of factors which influence the extent to which openness develops in cheese have been elucidated at this Institute(4) but, owing to the varying conditions from one cheese factory to another and in the same factory at different periods, the control of slit openness on a commercial scale constitutes a most difficult problem.

SUMMARY

"Open" cheese were shown to evolve carbon dioxide much more rapidly than did "close" cheese. From open cheese lactobacilli or betacocci capable of producing carbon dioxide relatively rapidly could be isolated. The addition of such organisms to cheese milk resulted in the development of slit openness in the cheese. The most common types causing slit openness appeared to be gas-producing lactobacilli.

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231. THE ASSESSMENT OF CURD FIRMNESS PRIOR TO CUTTING

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(With 2 Figures)

Scott Blair (1) has shown that the firmness of cheese curd at the stage immediately prior to cutting depends almost entirely on its rigidity or shear modulus. Other properties, such as degree of flocculation or contraction, are also of some importance in determining whether or not the curd should be cut, but these properties are correlated with firmness only in extreme cases.

With the aid of the apparatus designed by Scott Blair and described in his paper, a study has been made of the moduli of Cheddar, Cheshire, Double Gloucester and Derby curds during a period of one year. In order to test the reproducibility of the method, measurements were taken at random points in a vat during the course of hardening of a curd. Three tests were done on each spot. The values of ϵ obtained were as follows:

Table I

Min. after renneting	ϵ_1	ϵ_2	ϵ_3
41–45	1·47	1·46	1·47
50–54	1·39	1·40	1·40
63–68	1·30	1·28	1·32
72–76	1·29	1·29	1·30
80–84	1·24	1·25	1·27
88–92	1·25	1·25	1·25

As the curd remained uncovered for these experiments, it became abnormally cold, and it is not possible to judge at what point it would normally have been cut. The experiment shows that the method gives satisfactory reproducibility.

All the observations were made in the same dairy and an opinion was obtained from the same expert on each curd as to whether it was normal, firm or soft for that particular type of cheese. The expert had had considerable factory and teaching experience. The opinion was given as to firmness only, the firmness being judged mainly by pressing the curd with the back of the hand, though occasionally the opinion was influenced by the type of break when the curd was split with the finger. Additional observations made by the expert as to degree of flocculation or contraction were noted.

The aim of the present study was to determine the average firmness at which different curds are cut, to observe whether this changes with time of year and to test the reproducibility of subjective judgements of firmness.

Our expert informed us that she aims at cutting Cheshire and Double Gloucester curds at the same firmness. Cheddar curds should be somewhat firmer than this, and Derby curds somewhat softer. The ideal firmness for each type of curd is the same throughout the year.

In this experiment over 200 curds were tested. The mean of the readings taken on those Cheshire and Double Gloucester curds which were said to be of normal firmness was $1{\cdot}171 \pm 0{\cdot}0058$[1]; exactly the same figure ($\pm 0{\cdot}0064$) was obtained for the normal Cheddar curds. As the Double Gloucester curds were tested only in the winter months, it was not possible to compare the means for Cheshire and Double Gloucester separately. There was a slight difference in the means for normal and for soft Cheshire and Double Gloucester curds, the latter

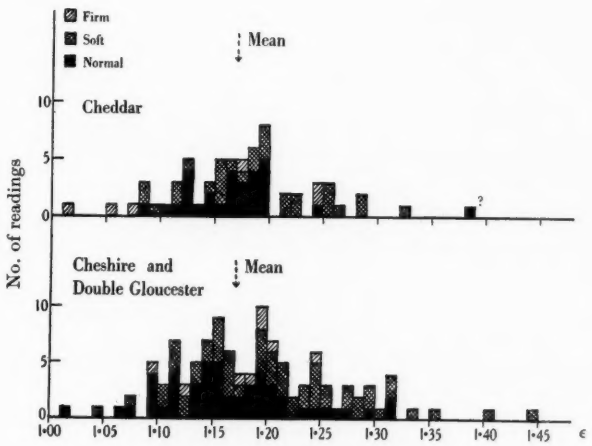

Fig. 1.

being $1{\cdot}210 \pm 0{\cdot}0083$, but without further data, this difference cannot be said to be statistically significant. This can be further seen from the histograms in Fig. 1. It is also clear from these diagrams that little or no objective significance can be attached to the judgement "too firm". In the diagram for the Cheshire and Double Gloucester curds only two of the "too firm" curds are below (i.e. harder than) the mode for the "normal" curds, whereas six come above it. It is interesting to note that there are far fewer "firm" than "normal" or "soft" judgements. This is partly because certain curds are incapable of becoming firm through some abnormal condition of the milk. It is probably also influenced by the fact that most soft curds are slightly flocculent, which may have led to the false assumption that most flocculent curds are soft. There seems to be no similar tendency to regard other factors, such as contraction, as correlated with too great a degree of firmness. It may

[1] Arbitrary units. Scott Blair's ϵ. These are the means of two readings on each curd.

be for the same reason that in the case of Cheddar, there are more "normal" readings below the mode than there are above it, though why this should be true only for Cheddar it is hard to say.

Fig. 2 shows the effect (not anticipated by the expert) of the season on judgements of firmness for all types of curd. Only two of those curds considered normal during the summer months fall below the mode of those considered normal in the winter months, the remaining twenty-two falling well above it. There are three possible reasons for this:

(1) A large number of the curds at this time were very weak as a result of some abnormality of the milk and the slightly soft ones may have seemed normal by comparison, but a similar state of affairs occurred during some of the winter months without any effect on the judgement of normal curds.

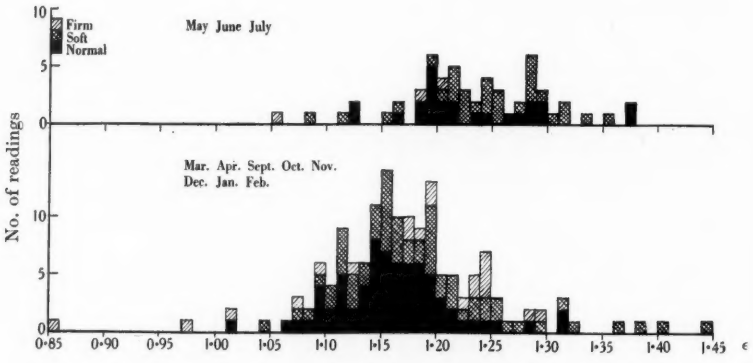

Fig. 2.

(2) The expert may have expected the curd to ripen and to "firm up" more quickly in the summer and this may have influenced her judgement.

(3) The summer weather and temperatures may have had some effect upon the actual sensitivity of the hand. We have some evidence that poor health, or worry has an adverse effect upon accuracy of judgement. On any one day, a difference in ϵ of about 0·09 corresponding to a difference in modulus of about 8% was generally correctly detected. This figure is almost the same as that expressing the smallest differences in the compression modulus of rubber cylinders which can be appreciated by the senses. (Work in process of publication.) On some days much greater differences were not observed. On several of these occasions the expert spontaneously reported that she was "off colour" or worried.

It is hoped to continue these experiments to see whether the seasonal effect is repeated, to discover its cause, and to obtain more accurate figures especially for the smallest difference in firmness which can be correctly observed. It is also hoped to extend observations to other dairies and other experts and to

find what variations in firmness can be allowed without detriment to the cheese. Similar experiments are being planned for the later stages in cheese manufacture.

SUMMARY

The reproducibility of Scott Blair's method for measuring curd firmness has been tested and measurements of the "firmness" of cheese curds immediately prior to cutting have been made and related to an expert's opinion.

Contrary to her beliefs it was found:

(1) that the mean firmness for normal Cheshire and Double Gloucester curds was the same as that for normal Cheddar curds,

(2) that normal curds, regardless of type, were softer in the summer months than in the winter.

Though confident opinions are often expressed on very small differences in firmness, the smallest difference in modulus which can be correctly appreciated appears to be of the order of 8%.

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232. A SIMPLE AND ACCURATE VISCOSIMETRIC FORM OF RENNET TEST

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(With 4 Figures and 4 Graphs)

Introduction and existing types of apparatus

In an investigation carried out during the summer of 1936 into the character of the mineral content of milks from several farms, with especial reference to calcium and phosphorus—an investigation which had the ultimate object of ascertaining whether any relationship obtained between the form in which these two elements were present and the facility with which cheese could be made from the milks in question—the necessity of employing an accurate form of rennet test was realized.

Exhaustive examination of the literature showed that at least four methods had been employed with more or less success, for which adequate descriptions were available. The authors of these tests were: (1) Mattick & Hallett(1); (2) Sommer & Matsen(2); (3) Christen & Virasoro(3); (4) Christen & Virasoro(4).

The principle of the Mattick & Hallett rennet test consists in noting the point of time at which the onset of coagulation coincides with the cessation of flow through a fine orifice of the milk containing the rennet, uniform temperature being maintained throughout. That of the Sommer & Matsen test marks the same instant by noting the formation of graininess inside glass bottles which have been partly filled with the milk containing the rennet and which are kept constantly agitated during the test, again at a uniform temperature.

The apparatus used by Christen & Virasoro was a rotating electro-magnetic viscosimeter and consisted of a weighted glass cylinder immersed in the liquid to be coagulated which was contained in a glass vessel fixed to a vertical axis, the containing vessel being immersed in a thermostat. The axis with the glass vessel was turned at a constant speed of 12 rev./min. during the experiment, and simultaneously the friction of the liquid produced a torsion couple on the weighted glass cylinder proportional to the viscosity of the liquid. The action of this couple was transmitted through a suspension rod to a static system made up of small permanent magnets. Two copper coils in series and placed perpendicularly to the magnets produced a field which tended to turn the static system in a direction opposite to that imparted to it by the liquid. In order to make a measurement the friction couple was adjusted by an electro-magnet,

that is, the system was brought into the initial position of equilibrium by regulating by means of a rheostat the current sent through the coils and the current strength read off on a milliammeter incorporated in the circuit.

Christen & Virasoro's experiments with this apparatus showed that the current strength is proportional to the viscosity of the liquid. For each viscosity determination the current strength was read off periodically at the beginning and more frequently as the current strength increased until finally it was impossible to take readings because of the too rapid variations. If the time in seconds be plotted against the corresponding relative viscosities, sets of graphs were obtained for each determination. The authors fixed as coagulation point the time corresponding to a relative viscosity of 2·6.

Finally, in 1935, Christen & Virasoro, in investigating the properties of rennin from vegetable sources, used a simplified form of the apparatus just described. This apparatus is shown in Fig. 1. The moving system consists of a brass tube T which turns between two ball-races C_1 and C_2 fixed to a support R. The containing vessel for the milk sample is fixed to T by a cork E. The system is turned at a constant speed of 12 rev./min. by a small electric motor. The torsion wire H (copper 0·1 cm. diameter) is attached to a hook K, and at its lower end by a small hook G to a weighted glass vessel B. L is a filter funnel placed over the exposed part of the torsion wire to protect against draughts while V is a thermostat. The torsion wire also carries a light glass pointer A which can be displaced along a horizontal scale graduated into one hundred divisions. In this apparatus a displacement of fifty divisions is taken as the coagulation point.

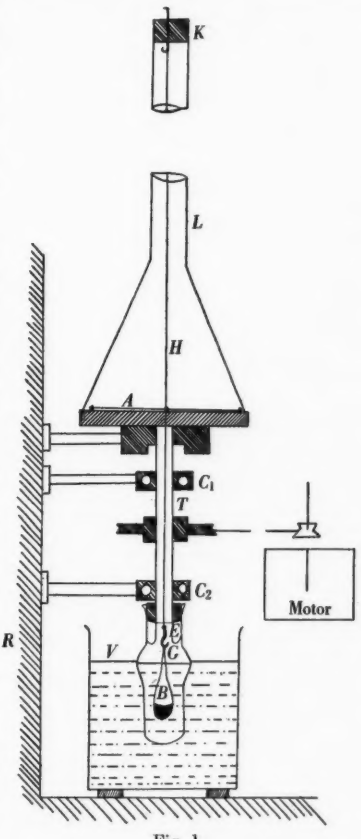


Fig. 1.

The following observations of Christen & Virasoro are worth quoting here; they ultimately influenced us in choosing a viscosity method:

> The method generally followed consisted in observing the formation of flakes. In quick rennet action the absolute error in measuring time is doubtless small but relative to the time of coagulation it becomes appreciable. In proportion as rennet tests were made with weaker rennet concentrations the determination of the exact moment of coagulation

becomes each time more difficult. The errors are still greater when one takes as coagulation point the moment when gelation has reached such a state that one can turn the test-tube upside down. We have, therefore, searched for a property which at the true moment of coagulation undergoes changes which can assist in fixing with precision this same instant. We have obtained good results with viscosity. An uninterrupted observation of the viscosity during the course of rennet action has shown us that from the time of adding rennet to the liquid on which it acts, this property remains practically constant until a certain instant where it increases in value accentuating strongly towards the end. There is no doubt that the coagulation points furnished by this method are more exact; they are comparable among themselves and are less subject to personal errors of observation.

Present type of apparatus

We have devised a piece of apparatus considerably less complicated than that described by Christen & Virasoro. In its ultimate and present form this apparatus is as follows:

An iron cylinder *A*, length 3 in., diameter 1 in., weight about 300 g., sprayed over with aluminium metal to prevent rusting, has fixed to one end of it a brass rod *C* which carries a brass disk *B* with light pointer *D* attached, all as shown in Fig. 2. The brass disk weighs about 200 g. and is 2 in. in diameter. The upper end of the brass rod *C* which passed through and above the disk *B* was split by a saw-cut and had a collar and screw placed over this to hold the edges of the saw-cut together so that a fine wire could be clamped between them. The upper end of the wire is attached to a fixed support as shown in the figure. The wire finally used was a steel wire 50 cm. long and 0·01 in. in diameter (violin E string). Consideration of G. E. Allan's(5) and G. Y. Zemplén's(6) work on the internal friction of gases suggested a suitable weight for the cylinder *A*, namely, 300 g.

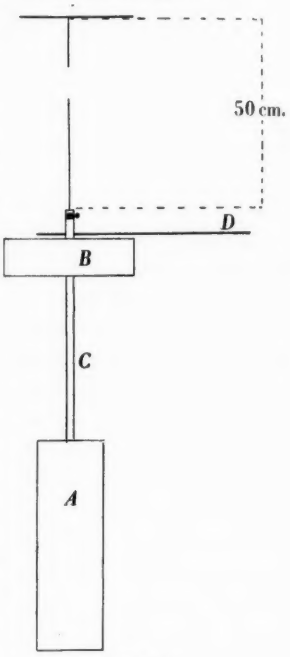


Fig. 2.

During an actual test the apparatus was assembled as shown in Figs. 3 and 4. The temperature of the water-bath was controlled by a small micro-burner: it was found that this provided an efficient thermostat. A 1 in 10 dilution of commercial rennet gave a suitable strength of rennet solution: this diluted solution must be freshly prepared.

A rennet test is carried out as follows: 250 ml. of milk in a beaker, heated to 40° C., are placed inside the water-bath which is maintained at the same temperature. The *A* part of the apparatus is then completely immersed in the

milk, care being taken to ensure that it does not touch any part of the containing beaker and can therefore turn freely. A stirrer so shaped that it can be moved round *A* and easily removed from the beaker is then placed in the milk. The pendulum is then brought to rest and 4 ml. of the diluted rennet solution added from a quick delivery pipette. When one half of the rennet has been added the stop-watch is started and the remainder then run in; the rennet is then very quickly mixed with the milk and the stirrer removed. The pointer *D* is now moved approximately 90° from its position of rest and the

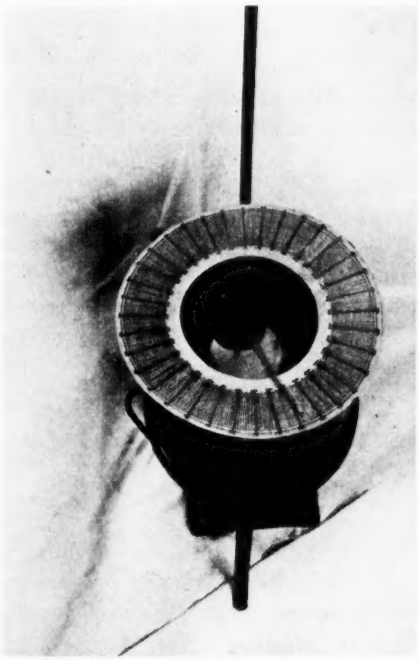


Fig. 3.

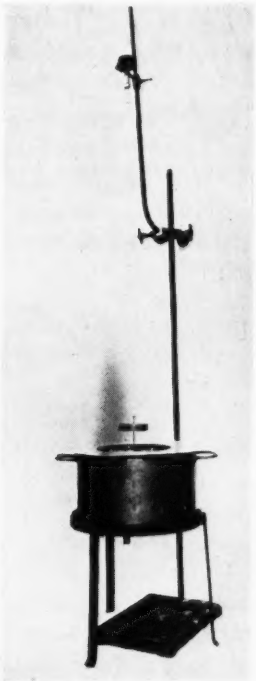


Fig. 4.

pendulum on release immediately begins to oscillate in the milk. The addition and mixing of the rennet, starting the pendulum to oscillate, occupy about 15 sec., and as far as possible this same time should be adhered to in carrying out these operations in all tests. The time of coagulation is taken as the time after the addition of one half of the rennet until the pendulum comes to rest.

RESULTS OBTAINED WITH PRESENT TYPE OF APPARATUS

The experimental conditions for carrying out a rennet test as just described indicate, of course, that the method is empirical and that to obtain results comparable not only as between duplicates but also as between estimations

with different milks it is necessary to adhere to a well-defined procedure in carrying out the tests. Table I records some earlier results obtained before a rigid procedure had been defined and adhered to, and it illustrates this point very well.

Table I

250 ml. milk at 40° C. 4 ml. rennet solution added. Wire length 50 cm. Number of twists given to wire 2, all in every test.

Coagulation times									
Sample 1		Sample 2		Sample 3		Sample 4		Sample 5	
min.	sec.	min.	sec.	min.	sec.	min.	sec.	min.	sec.
2	58	3	11	3	20	2	32	4	32
3	8	3	4	3	11	2	47	4	31
3	1	3	5	3	15	2	30	5	24
3	0					2	36	(Water added)	

This table shows a variation between the extremes in each sample, excluding sample 5 where conditions were materially altered by the addition of water in the last test, ranging from 7 to 17 sec. In the next set of investigations the conditions were made as exactly similar as possible and the procedure expressly stated above strictly followed out. Table II records these results.

Table II

250 ml. milk at 40° C. 4 ml. rennet solution added. Wire length 50 cm. Twisted 90° only from rest, all in every test. Trials made with two different dilutions of rennet solution.

Coagulation times			
Rennet diluted 1 in 4		Rennet diluted 1 in 10	
min.	sec.	min.	sec.
1	16	4	32
1	12	4	31
1	12		
1	13		

At this point we should like to draw attention to the nature of the surface of the *A* part of the pendulum. As previously mentioned, this had been sprayed over with aluminium metal to prevent rusting. We strongly recommend that this aluminium surface be left in a rough state. At one time we thought that the rough surface would be a disadvantage in being less easily kept clean, and did not consider that the surface of *A* would have any bearing on the efficiency of the pendulum. Accordingly the cylinder was polished and Table III records the results obtained.

Table III

250 ml. milk at 40° C. 4 ml. rennet solution added. Wire length 50 cm. Number of twists given to wire 2, all in every test.

Coagulation times			
Sample 1		Sample 2	
min.	sec.	min.	sec.
3	52	4	22
4	38	4	30
4	0	4	2
3	0	4	5

The only explanation of the above unsatisfactory results seemed to lie in the surface of the cylinder. It was thought that the curd did not attach itself to the polished surface of the cylinder and as the whey exuded it acted as a lubricant between the surfaces of the curd and cylinder. The cylinder was then again sprayed and the surface left rough. It is interesting to note here that similar discrepancies arose during the ensuing summer's work and were traced to the surface of the cylinder becoming coated with fat derived from the milk. The condition of the cylinder surface *A* is thus very important for the agreement of duplicates, and periodically the cylinder should be washed in petroleum ether to get rid of any adhering fatty material.

In order to test out the apparatus further, before using it in the summer's work, opportunity was taken to carry out rennet tests on cheese milk during the process of ripening before the addition of the rennet. The experiment was begun immediately after all the milk had been added to the vat. 250 ml. milk at 40° C. and 4 ml. diluted rennet were used as in the previous experiments. The results obtained are shown in Table IV.

Table IV

Time of test a.m.	Time of coagulation min.	sec.
7.35	2	38
7.50	2	20
8·05	2	16
8.20	2	11
8.35	2	6
8.50	1	59
9·05*	1	50

* Rennet added to the milk in the vat at this point

Finally, Table V records results obtained with several different samples of milk during the summer's work. Results are duplicated.

Table V

250 ml. milk at 40° C. 4 ml. rennet solution added. Wire length 50 cm. Twisted 90° from rest, all in every test.

Sample no.	Coagulation times min.	sec.	min.	sec.
1	2	49	2	45
2	2	40	2	41
3	3	31	3	40
4	3	0	3	5
5	3	13	3	5
6	4	0	3	54
7	2	58	3	1
8	2	46	2	46
9	2	50	2	57
10	3	21	3	25
11	3	19	3	24
12	3	15	3	17
13	3	41	3	47
14	2	37	2	42

MATHEMATICAL TREATMENT OF THE PROBLEMS INVOLVED

The action of the pendulum viscosity apparatus in relation to milk containing rennet was now studied with the precise aim of ascertaining how far the practical results obtained in the rennet coagulation of milk, as determined with the apparatus, accorded with the results which would be expected from its use from the theoretical standpoint. This part of the investigation showed such a close agreement between theory and practice, as to establish firmly the scientific soundness of the apparatus, that we think it of some advantage to include an account of it here.

Without going into the details of the theory underlying oscillatory motion and viscosity—a subject fully treated in the appropriate textbooks(7)—we shortly state here that in the case of a torsional pendulum the motion may be regarded as simple harmonic, the restoring force being proportional to the angular displacement, and if the pendulum be vibrating in a gas or liquid the resistance is proportional to the velocity of movement. Also, when adjacent layers of a fluid move with a relative velocity, viscous forces are brought into play tending to reduce the relative movement. Finally, the viscosity of a liquid may be determined by timing the period of oscillation of a flat circular disk and finding the logarithmic decrement (the meaning of which can also be gleaned from the textbooks) in the liquid and in air. O. E. Meyer(8) has shown that with such a disk the coefficient of viscosity for a liquid, η, is:

$$\eta = \frac{16I^2}{\pi\rho T\,(r^4+2r^3\,d)^2}\left\{\left(\frac{\lambda-\lambda_0}{\pi}\right)+\left(\frac{\lambda-\lambda_0}{\pi}\right)^2\right\}^2,$$

where I is the moment of inertia of the disk and attachments about the axis of suspension; ρ is the density of the liquid; T is the time of a complete swing in air, i.e. the periodic time; r is the radius of the disk; d is the thickness of the disk; λ is the logarithmic decrement in the liquid; λ_0 is the logarithmic decrement in air.

It can also be shown(7) that the periodic time, T, of such oscillation is

$$T = 2\pi/\sqrt{(F/I - k^2/4I^2)},$$

where F is the restoring force per unit angular displacement and k is the frictional resistance.

Also it can be shown(7) that the amplitude, i.e. the amount of angular displacement, is $\theta_0\, e^{-kt/2I}$, and decreases exponentially with the time. θ_0 is the amplitude when the friction is eliminated, i.e. when $k=0$.

In the case where the frictional resistance, k, is very small, the value of k^2 is correspondingly much smaller and $k^2/4I^2$ becomes negligible compared with F/I. The periodic time then becomes

$$T = 2\pi\sqrt{(I/F)}.$$

The oscillating body therefore performs vibrations in equal times, but the amplitude gradually dies out.

Starting from the undisturbed position of the body, let α_1 be the angular displacement measured to the first turning point (to the right, say). Let α_2 be

the angular displacement following on the other side of zero (to the left, say): also measured from the zero position. Let α_3 be the next swing in the original direction from the zero.

The first deflexion α_1 is after a time $t=T/4$; α_2 after a time $3T/4$; α_3 after a time $5T/4$, ...,

$$\text{To the right } \alpha_1=\theta_0\, e^{-k/2I\,T/4},$$
$$\text{To the left } \quad a_2=\theta_0\, e^{-k/2I\,3T/4},$$
$$\text{To the right } \alpha_3=\theta_0\, e^{-k/2I\,5T/4},$$
$$\text{To the left } \quad \alpha_4=\theta_0\, e^{-k/2I\,7T/4}.$$

Thus $$\alpha_1/\alpha_2=e^{-k/2I\,T/2}=\alpha_2/\alpha_3=\alpha_3/\alpha_4=\alpha_4/\alpha_5=\alpha_n/\alpha_{n+1},$$

so that $$\log \alpha_1/\alpha_2=\log \alpha_3/\alpha_4=\log \alpha_5/\alpha_6=\log \alpha_n/\alpha_{n+1}=k/2I\ T/2=\lambda.$$

$\lambda=k/2I\ T/2$ is called the logarithmic decrement.

Again, $$\frac{\alpha_1}{\alpha_2}=\frac{\alpha_2}{\alpha_3}=\frac{\alpha_3}{\alpha_4}=\ldots=\frac{\alpha_n}{\alpha_{n+1}},\ \therefore\ \log\left(\frac{\alpha_1}{\alpha_2}\frac{\alpha_2}{\alpha_3}\frac{\alpha_3}{\alpha_4}\ldots\frac{\alpha_n}{\alpha_{n+1}}\right)=\log\left(\frac{\alpha_1}{\alpha_2}\right)^n,$$

whence $$n\log\frac{\alpha_1}{\alpha_2}=\log\frac{\alpha_1}{\alpha_{n+1}};$$

and as $$\lambda=\log\frac{\alpha_1}{\alpha_2},\quad \therefore\ \lambda=\frac{1}{n}\log\frac{\alpha_1}{\alpha_{n+1}}.$$

Further, since $$\frac{\alpha_1}{\alpha_2}=\frac{\alpha_2}{\alpha_3},\quad \therefore\ \frac{\alpha_1}{\alpha_2}=\frac{\alpha_1+\alpha_2}{\alpha_2+\alpha_3};$$

similarly $$\frac{\alpha_2}{\alpha_3}=\frac{\alpha_2+\alpha_3}{\alpha_3+\alpha_4},$$

$$\therefore\ \log\frac{\alpha_1}{\alpha_2}=\frac{1}{n}\log\left(\frac{\alpha_1+\alpha_2}{\alpha_2+\alpha_3}\frac{\alpha_2+\alpha_3}{\alpha_3+\alpha_4}\ldots\frac{\alpha_n+\alpha_{n+1}}{\alpha_{n+1}+\alpha_{n+2}}\right),\ \text{and}\ \lambda=\frac{1}{n}\left(\frac{\alpha_1+\alpha_2}{\alpha_{n+1}+\alpha_{n+2}}\right).$$

If, for convenience, $\alpha_1+\alpha_2$ be replaced by β_1, $\alpha_2+\alpha_3$ by β_2 ..., $\alpha_n+\alpha_{n+1}$ by β_n ..., then the equation for λ becomes

$$\lambda=\frac{1}{n}\log\frac{\beta_1}{\beta_{n+1}},\ \text{ or }\ \lambda=\frac{1}{n-1}\log\ \frac{\beta_1}{\beta_n}.$$

The method of finding λ set out in the above equation depends on the observation of swing from left to right ($\frac{1}{2}$ periods), and no knowledge of the zero reading is required. The number of complete swings may be obtained by dividing the total time by T the periodic time; hence n and $(n-1)$ are known.

With milk under the action of rennet a series of logarithmic decrements is obtained and when the logarithmic decrements are plotted against time a curve is obtained showing the changes in logarithmic decrement. From this curve, using a modified form of the formula already given for the coefficient of viscosity (such modification being derived from the facts that λ_0 is very small compared with λ and can be neglected, and λ is small in comparison with π, so $(\lambda/\pi)^2$ can also be neglected), viz.

$$\eta=\frac{16I^2}{\pi\rho T\ (r^4+2r^3d)^2}\left\{\frac{\lambda}{\pi}\right\}^2,$$

a curve showing the change in viscosity coefficient under the action of rennet is obtained.

Now we sought to determine in actual practice the diminishing amplitude values $\alpha_1+\alpha_2 \ldots \alpha_n+\alpha_{n+1}$ as above defined. The apparatus was assembled with the scale and pointer arranged as shown in Figs. 3 and 4. The scale, consisting of a large circular disk divided into degrees, was placed so that the oscillating wire was in the centre of its circle and the pointer as close to its graduations as possible while still moving freely.

The periodic time of the pendulum was observed by counting twenty consecutive half-swings and was found to be 7 sec. in air, 7·2 sec. in milk and 7·2 sec. in water.

The pendulum was then allowed to oscillate in milk containing rennet and the readings taken at each turning point of the pointer on the scale. From these readings $\alpha_1+\alpha_2 \ldots \alpha_n+\alpha_{n+1}$ were obtained. The results are shown in Table VI. From these readings the logarithmic decrements were calculated, using the formula

$$\lambda=\frac{2\cdot303}{n-1}\log_{10}\frac{\beta_1}{\beta_n}.$$

Table VI. *Cylinder as part of pendulum*

Time periods	$\alpha_1+\alpha_2 \ldots \alpha_n+\alpha_{n+1}$	Logarithmic decrement*	Viscosity coefficient*	Time periods	$\alpha_1+\alpha_2 \ldots \alpha_n+\alpha_{n+1}$	Logarithmic decrement*	Viscosity coefficient*
1	298			34	131		
2	293	0·02695	0·02599	35	129		
3	286			36	125		
4	284			37	122		
5	278			38	119		
6	265			39	116		
7	258			40	113		
8	253			41	110		
9	—			42	107		
10	234	0·02535	0·02295	43	103		
11	227			44	100		
12	222			45	98	0·02027	0·01471
13	218			46	95		
14	213			47	91		
15	207	0·01911	0·01306	48	88		
16	202			49	86		
17	198			50	84	0·02349	0·01974
18	193			51	80	0·04880	0·08509
19	188			52	74	0·10000	0·3579
20	184	0·02188	0·01713	53	68		
21	180			54	58	0·1589	0·9038
22	175			55	43	0·37613	5·062
23	171			56	30		
24	166			57	23	1·0208	
25	162	0·02441	0·02132	58	18		
26	160			59	13		
27	157			60	8		
28	153			61	5		
29	149			62	4		
30	145	0·02741	0·02689	63	3		
31	141			64	2		
32	138			65	1		
33	134						

* Points chosen where the amplitude curve obviously changed in slope.

Then the viscosity coefficients were calculated from these results, using the modified formula shown above.

The cylinder which we employed was very convenient for use with milk, but in conducting viscosity experiments on other liquids a flat disk is commonly used as vibrator, and it was considered advisable to go through the experiments, as described in this section, again with a disk in order to obtain confirmation.

Table VII. *Disk as part of pendulum*

Time periods	$\alpha_1+\alpha_2 \ldots \alpha_n+\alpha_{n+1}$	Logarithmic decrement*	Viscosity coefficient*	Time periods	$\alpha_1+\alpha_2 \ldots \alpha_n+\alpha_{n+1}$	Logarithmic decrement*	Viscosity coefficient*
1	210			30	69	0·04261	0·03147
2	200	0·035466	0·02179	31	66		
3	193			32	64		
4	188			33	61		
5	180			34	59		
6	174			35	57	0·03050	0·01613
7	169			36	55		
8	160			37	53		
9	155			38	50		
10	144	0·04076	0·02879	39	48		
11	138			40	47	0·04168	0·03011
12	134			41	45		
13	128			42	43		
14	123			43	41		
15	119	0·03216	0·01793	44	39	0·05274	0·04820
16	115			45	36		
17	110			46	34		
18	107			47	32		
19	103			48	30		
20	99	0·03961	0·02719	49	27		
21	95			50	23	0·16052	0·4463
22	92			51	18		
23	89			52	14		
24	86			53	10		
25	83	0·03547	0·02181	54	6	0·51081	5·697
26	81			55	5		
27	77			56	3		
28	75			57	2		
29	73			58	1		

* Points chosen where the amplitude curve obviously changed in slope.

Notes on Tables VI and VII

Examination of these tables reveals the fact that the logarithmic decrements, and derived from these the viscosity coefficients, vary in a remarkable manner, the increase in values being by no means consistent throughout the period the rennet is acting on the milk. This is also, of course, brought out by the graphs. This point is under further consideration and investigation.

It should also be made clear that the concluding values for logarithmic decrement and viscosity coefficient in both tables, after the point of time where they reach significant figures in the first place of decimals, at some point refer to a medium which is ceasing to be a true fluid—milk for all practical purposes being regarded as a true fluid—but passing through semi-fluidity into a state where shear and stress conditions must considerably modify the values actually obtained. In this connexion see also observations on the graphs referring to the branching shown in the "Dying away of the amplitude curve" (observations nos. 3–6).

Accordingly, a flat brass disk was obtained with approximately the same moment of inertia as the cylinder. The radius and depth of the disk were 2·5 and 0·5 cm. respectively as compared with the corresponding figures of 1·35 and 7·5 cm. for the cylinder. It was sprayed over with aluminium metal to give a surface similar to that of the cylinder. The periodic time of the pendulum

in air when the cylinder was replaced by the disk was 7·5 sec. The pendulum was again allowed to oscillate in milk containing rennet and the readings obtained are set forth in Table VII. It is to be noted that the same milk was used for the two sets of readings recorded in Tables VI and VII. Graphs were also constructed from these readings and it is noteworthy to remark on the similarity of the curves obtained.

Observations on the graphs

In studying the graphs we draw attention to the following points:

(1) The units marked along the X axis, i.e. abscissae, are in terms of the time taken for a single one-half periodic oscillation, and these one-half periodic oscillations are taken consecutively. In the case of the cylinder pendulum this unit amounts to $\frac{1}{2}$ (7·0) sec., i.e. 3·5 sec.; and $\frac{1}{2}$ (7·5) sec., i.e. 3·75 sec. in the case of the disk pendulum.

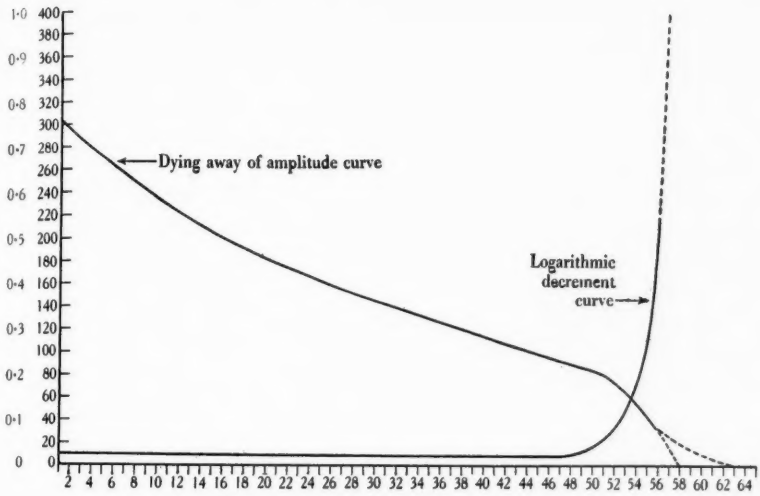
(2) Two scales appear on the Y axis, i.e. ordinates, where two graphs are shown on the same sheet, viz. the whole numbers referring to the "Dying away of amplitude curve" and the fractional numbers to the logarithmic decrement curve.

(3) Particular attention is directed to the close of the "Dying away of amplitude curve" where a branch in the curve is shown, both branches appearing as dotted lines. The upper dotted branch represents the plotting of the values obtained when coagulation has visibly set in and the movements of the cylinder and/or disk are becoming irregular and are not really oscillatory movements. This irregularity of movement, in our opinion, makes the values here obtained of doubtful accuracy and accordingly this part of the curve has been shown as dotted. The lower branch is merely a continuation of the direction of the main curve and is thus also imaginary and hence it has been shown dotted as well.

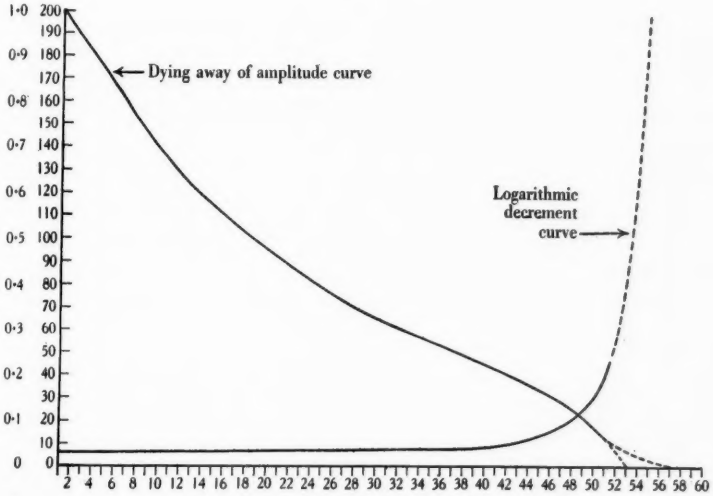
(4) From (3) we recommend that the true point for determining onset of coagulation be taken as that where the branching therein described begins, viz. 56 for the cylinder curve and 51·5 for the disk curve.

(5) Accordingly, from (4) the total time from addition of rennet to onset of coagulation as shown by the cylinder curve is $56 \times 3{\cdot}5$ sec. $= 196$ sec., and as shown by the disk curve is $51{\cdot}5 \times 3{\cdot}75$ sec. $= 193$ sec.: this is a remarkable degree of agreement.

(6) Similarly, as the logarithmic decrement curve is derived from the values for the "Dying away of amplitude curve," the former is continued as a dotted line after the time units specified for the cylinder and disk oscillations, namely, 56 and 51·5 respectively. On the other hand, as the values for viscosity coefficients suddenly become much larger at these points and in fact cannot be plotted within the limits of the graph page, the curves for viscosity coefficients are unbroken lines.

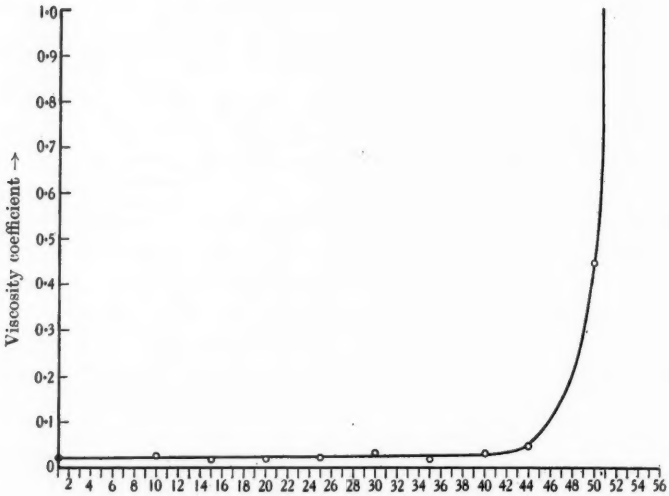


Cylinder pendulum.



Disk pendulum.

Cylinder pendulum. Viscosity coefficient curve.



Disk pendulum. Viscosity coefficient curve.

We make these further observations arising out of the graphs:

(1) The ideal method for carrying out a rennet test by the present type of apparatus would be to take observations, as just detailed, during the time of its progress and plot at least the "Dying away of amplitude curve" and note the time unit where branching occurs.

(2) Since time, however, will not probably allow for this procedure, it is indicated that a more accurate method for marking the end-point of the rennet test would be to take such an end-point as that instant where the pointer *D*, in the course of its diminishing oscillations, dies down to a certain small angle on the circular scale. We are indebted to Dr G. E. Allan for this suggestion, and it is interesting to record that while Dr Allan hitherto has not seen the graphs, the "Dying away of amplitude curve" where it branches is in support of this idea. This suggestion is really an amendment of the closing stage of the procedure recommended earlier in this paper for conducting a rennet test with the present type of apparatus.

SUMMARY

1. Some existing types of rennet tests are briefly reviewed.

2. A viscosity type of rennet test is described and results from its use are given.

3. A mathematical treatment of the problems involved is given, which leads on to

4. Construction of graphs showing progressive variation in "Dying away of amplitude", "Logarithmic decrement" and "Viscosity coefficients" of milk under the action of rennet. Since viscosity determinations on liquids and gases have been commonly carried out with a disk type of viscosimeter, such a type is now introduced and similar graphs constructed from observations during its use. A close similarity in graphs to those furnished by the cylinder type of viscosimeter is obtained. The cylinder type of apparatus, we find, is more convenient in use with milk in reference to rennet action.

5. Arising from the graphs a more precise method of terminating a rennet test with the present type of apparatus is indicated.

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BIENNIAL REVIEWS OF THE PROGRESS OF DAIRY SCIENCE

SECTION F. MILK-BORNE DISEASES

637.128:616.9

THE milk supply and its relation to public health continues to be a topic in which much interest is displayed. Agreement has been reached on two points—that the milk supply should be clean and safe, and that the young in particular should consume a larger quantity. The control of milk-borne diseases resolves itself into three distinct aspects: first, the control of disease in animals; secondly, the control of infections and carrier conditions amongst the personnel engaged in the dairying business; and thirdly, pasteurization. It seems reasonable to believe that the combination of all three methods would produce a safe milk supply. Recent papers on the effect of pasteurization on the nutritive value of milk give no indication of any marked deleterious action. Again, Bendixen *et al.*(1), in a report to the Health Organization of the League of Nations on all aspects of the milk problem, state that it is their considered emphatic opinion that all liquid milk for human consumption should be adequately pasteurized, or boiled. Furthermore, Wilson(2), in a critical review of the position as regards pasteurization, asserts that, so far as available evidence goes, there is no scientific argument against pasteurization, provided minor additions are made to the dietary of infants whose sole source of nourishment is pasteurized cows' milk. He concludes that there is every reason to believe that the introduction of compulsory pasteurization carried out under proper supervision would lead to the practical abolition of milk-borne diseases. This subject has also been discussed by Minett & Pullinger(3), and by Savage *et al.*(4).

I. STREPTOCOCCAL INFECTIONS

The pyogenic streptococci continue to play a prominent part in milk-borne epidemic disease. Accordingly, a proper classification of these organisms is essential to an understanding of the part which the various types may play in disease processes in both animals and man. Sherman(5) has reviewed the literature on streptococci, and has produced a tentative classification based on modern methods of study as shown in the table on p. 356.

As regards morphology of the organism, Hobby & Dawson(6) have shown that if strains of haemolytic streptococci in the mucoid phase are subcultured into broth the capsules can be demonstrated after a growth period of 3–4 hr. Various strains of *Streptococcus pyogenes* in Lancefield's group A–F have been found to be capsulated, and furthermore, group A haemolytic streptococci when first isolated from disease processes in the human are usually capsulated and in the mucoid phase. The Lancefield method of grouping the streptococci continues to show valuable results, and so also the Griffith agglutination technique for identifying strains within group A. Fuller(7) described a new method of extracting the group-specific substance by the use of formamide, and believes that it has the advantage over other methods in completely dissolving the bacteria, thereby giving potent extracts, and of destroying or removing protein substances which might give cross-reactions. The method is as follows: 5 c.c. of an 18 hr. glucose-broth culture are spun down in the centrifuge, and the supernatant is removed as completely as possible. 100 c.mm. of formamide are

Division of the Streptococci

Division	Group or species	Lancefield group	Haemolysis	Growth at 10° C.	Growth at 45° C.	Growth in presence of 6·5% NaCl	Growth in presence of *p*H 9·6	Growth in presence of 0·1% methylene blue	Strong reduction	Survival 60° C., 30 min.	NH_3 from peptone
Pyogenic	*Str. pyogenes*	A	+	−	−	−	−	−	−	−	++
	Str. mastitidis	B	±	−	−	−	−	−	−	−	
	Str. equi	C	+	−	−	−	−	−	−	−	·
	"Animal pyogenes"	C	+	−	−	−	−	−	−	−	+
	The "human C"	C	+	−	−	−	−	−	−	−	+
	"Minute haemolytic"	F	+	−	−	−	−	−	−	−	+
	Group G streptococci	G	+	−	−	−	−	−	−	−	+
	Group E streptococci	E	+	−	−	−	−	−	−	−	+
	Group H streptococci	H	±	−	+	−	−	−	−	±	±
Viridans	*Str. salivarius*		−	−	±	−	−	−	−	−	−
	Str. equinus		−	−	+	−	−	−	−	±	−
	Str. bovis		−	−	+	−	−	−	−	+	−
	Varieties of *Str. bovis*		−	−	+	−	−	−	−	+	−
	Str. thermophilus		−	−	+	−	−	−	−	+	−
Lactic	*Str. lactis*		−	+	−	−	−	+	+	±	+
	Str. cremoris		−	+	−	−	−	+	+	±	−
Enterococcus	*Str. faecalis*		−	+	−	+	+	+	+	+	+
	Str. liquefaciens		−	+	+	+	+	+	+	+	+
	Str. zymogenes	D	−	+	+	+	+	+	+	+	+
	Str. durans		+	+	+	+	+	+	−	+	+

added to the residue, and the tube shaken and placed in an oil bath at 150° C. for 15 min. The tube is allowed to cool, and 250 c.mm. of acid alcohol are added, mixed, and the precipitate removed by centrifuging. The supernatant is drawn off by pipette, placed in a small tube, and 500 c.mm. of acetone added. The tube is shaken, the precipitate spun down and the supernatant discarded. The acetone precipitate is very small in amount, but it contains practically all the group antigen. 1 c.c. of saline and a drop of phenol-red indicator are added to the precipitate, and the whole neutralized with a trace of sodium carbonate. This solution is used for the test.

It is now believed that group A strains of haemolytic streptococci are responsible for the vast majority of streptococcal infections in man. Scarlet fever, tonsillitis, and erysipelas must be entirely due to this type, and so also most cases of septicaemia arising from infected uteri and wounds. It will be shown later, however, that these group A strains can be recovered from the bovine udder, and Edwards (8) has isolated them from the respiratory tract of chicks with bronchitis. Group B strains, usually associated with disease of the bovine udder, have been recovered from the human throat and nose (Hare (9), Plummer (10)), human vagina (Lancefield & Hare (11)), human faeces (Smith & Sherman (12)), and from two cases of human septicaemia by Fry (13). The "C" types are now divided into three subtypes, which appear to have characteristic sugar reactions, as follows (Sherman (5)):

	Lactose	Trehalose	Sorbitol
C. Human	±	+	−
C. *Str. equi*	−	−	−
C. Animal pyogenes	+	−	+

The human "C" strains have been isolated from throat, nose, vagina and skin. There is no indication that this organism is of much importance in human infections. *Str. equi* does not appear to have been isolated from human sources, and so also the "C" animal *pyogenes* strain which is the cause of a variety of infections in lower animals. Group D strains have been isolated from the human intestine and vagina, group E from the milk of cows, and groups F, G and H strains from the human throat, faeces or vagina. There is little evidence that they produce any disease process.

It is now believed by most workers that the majority of milk-borne epidemics arise not from direct contamination from a human source but by contamination from a cow suffering from mastitis due to haemolytic streptococci of human origin. The importance of grouping strains of haemolytic streptococci isolated from the bovine udder by the Lancefield technique cannot therefore be overestimated. That group A strains are infrequently present in milk has been shown by Sherman & Niven (14), who examined 313 samples of raw and pasteurized milk. These workers found that 8·5% of pasteurized and 18% of raw milk showed the presence of various types as follows:

Lancefield group B	24 strains	*Str. mastitidis*	
,, C	11 ,,	Animal *pyogenes*	
,, D	6 ,,	*Str. pyogenes*	Heat-resisting streptococci of pasteurized milk
,, D	19 ,,	*Str. durans*	
Others	4 ,,		

That strains of haemolytic streptococci from human sources are capable of infecting the bovine udder experimentally has been clearly shown by Davis (15), who, when he applied such strains to teat surfaces, found that they would readily ascend the teat ducts. Recently, Bendixen & Minett (16) have recorded their observations on two cows which were responsible for milk-borne epidemics, one in Denmark and the other in England. The Danish cow excreted group A haemolytic streptococci for 13 months, excluding a dry period of 3 months, while the cow involved in the Doncaster epidemic excreted the streptococci for 6 weeks. In both cases there were

stages when symptoms of mastitis were either not evident or not pronounced, although the milk was known to be infective. Again, the findings of Pullinger & Kemp(17) also point to the fact that the infected cow must be mainly responsible. These workers studied the growth of *Str. pyogenes* in raw milk stored at atmospheric temperature, 15–18° C., and found that, first, there is a latent period of 48–72 hr. before the streptococci commence to multiply, secondly, that raw milk exerts a definite bactericidal action, and thirdly, that the presence of saprophytic bacteria exerts an inhibiting action.

Various epidemics due to *Str. pyogenes* continue to be reported. These occur in three forms, scarlet fever, epidemic sore throat, and mixed outbreaks of scarlet fever and sore throat. It would seem that the nomenclature of human streptococcal infections requires revision. At the present time no specific difference can be detected in many strains of haemolytic streptococci causing scarlet fever in some individuals and tonsillitis in others, while certain strains produce sore throat but no scarlet fever. It would appear that, to cause scarlet fever, the strains must produce erythrogenic toxins in the throat of an individual who has a minimum amount of antitoxin, whereas in the individual with that amount of antitoxin necessary to neutralize the absorbed toxins only tonsillitis will be evident. On the other hand, certain strains of *Str. haemolyticus* appear to excrete relatively weak toxins, yet be capable of causing tonsillitis. In this instance, individuals who give a positive Dick test reaction will develop tonsillitis as well as those with a Dick negative test. The best way to review the recent literature will be to consider those epidemics occurring in the U.S.A. first, and those reported from other countries later. The *U.S. Public Health Service Reports* give the following:

	1936			1937		
	Epidemics	Cases	Deaths	Epidemics	Cases	Deaths
Scarlet fever	12	971	16	11	324	3
Scarlet fever and septic sore throat	—	—	—	1	312	0
Septic sore throat	6	268	7	2	350	0

In 1936 the most severe epidemic, scarlet fever in type, occurred at Owego, N.Y., where, in a population of 4891 individuals, some 511 contracted the disease from raw milk. It was believed that a cow suffering from mastitis was responsible for the outbreak. In 1937 a further large outbreak of 645 cases of septic sore throat occurred at West Des Moines, Iowa, in a population of 4400. It was proved that at least 312 individuals had consumed milk from a supply which had been infected with haemolytic streptococci from a bovine source. In the thirty-two outbreaks in 1936 and 1937, at least twelve were believed to be due, in part at least, to bovine carriers.

In this country no further serious outbreak has occurred since that described in the previous review by Scott(18), and by Watson(19). This latter epidemic was an example of the mixed type of infection in which cases of scarlet fever and sore throat were both observed. Leusden(20) described an epidemic of scarlet fever which occurred at Penneberg in Germany. In all, there were 450 cases, with eight deaths. The outbreak was traced to a milker who had been in contact with a child with scarlet fever, and the organism causing the epidemic belonged to type 5. Again, Henningsen & Ernst(21) described an outbreak of 100 cases of sore throat in a small town of 750 inhabitants in Denmark, due to Lancefield group A haemolytic *Streptococcus*. The infection was traced to milk which had been infected by a cow suffering from mastitis due to the same type of haemolytic *Streptococcus*. Finally, in a recent review on bovine mastitis by Munch-Petersen(22), a section is devoted to the relationship of streptococcal mastitis to human health. The literature up to the end of 1935 dealing with four aspects of the problem, namely, (1) epidemics, (2) organisms concerned with milk-

borne epidemics due to mastitis, (3) pasteurization, and (4) streptococci in market milk, is summarized.

II. *Staphylococcus* infections

Considerable interest continues to be shown in staphylococcal food poisoning, and consequently in staphylococcal infections of cows and the contamination of milk and milk products.

The classification of the staphylococci has not reached finality. Differentiation based on pigment production and fermentation reactions has not been successful, since the definite *aureus* and *albus* varieties show intermediate forms, although so far as fermentation reactions are concerned, the production of acid in mannitol is more often associated with *aureus* than *albus* strains. It would appear probable, however, that the *citreus* types can be differentiated from *Staph. aureus* and *Staph. albus*. There seems to be now sufficient evidence, however, to show that *aureus* and *albus* varieties can be further classified on the basis of pathogenicity and toxin production. It has been demonstrated that *aureus* strains produce soluble toxins with haemolytic, leucocidal, necrotizing, lethal and enterotoxic properties. That more than one factor is involved in the production of these reactions has been proved by Glenny & Stevens (23), who found that certain strains produced two toxins. This work has been further elaborated by Morgan & Graydon (24) and Smith & Price (25, 26), who described a third haemolytic toxin. A summary of the main features of this work is given in the following table:

	α toxin	β toxin	γ toxin
Sheep cells	Haemolysis in water bath	Sometimes slight haemolysis at 37° C.; rapid haemolysis on cooling	Haemolysis
Rabbit cells	Haemolysis	No haemolysis	Haemolysis
Intracutaneous injection of guinea-pigs	Necrosis	No necrosis	Slightly dermonecrotic
Intravenous or intraperitoneal injections into mice	Lethal	Non-lethal	Non-lethal
Intravenous injections into rabbits	—	Lethal in large animals	Lethal

In addition it has been found possible to produce a specific antitoxin to each of the above factors. Certain strains have also been found to be capable of forming in culture media, and under natural conditions, a heat-resisting toxin which, when ingested by human beings, causes definite signs of food poisoning. The work of Jordan, Dack and their co-workers on staphylococcal food poisoning has received further corroboration, while the method devised by Dolman *et al.* (27) for the detection of the enterotoxic substance by the inoculation of filtrate intraperitoneally into kittens has been found to be practical and satisfactory. Minett (28) has carried out an extensive investigation into the properties of this enterotoxin. He has found it to be resistant to heat (95° C. for 30 min.), to low concentrations of formalin sufficient to destroy the haemolytic toxins, to an acidity of *p*H 5·0, and to the action of rennet. It was found to be destroyed by trypsin. It was readily produced under suitable cultural conditions, and it is definitely antigenic. Finally, this worker states that its properties are such that it can be classified as a bacterial exotoxin. Since Dolman in his work with kittens removed the non-enterotoxic factors by boiling and by treatment with formaldehyde, Davison *et al.* (29) believed that the enterotoxic factor might perhaps also be partially destroyed. These workers carried out an investigation on the neutralizing action of immune sera. They utilized a non-enterotoxic strain of *Staphylococcus* which elaborated the haemolytic, dermo-necrotic and lethal toxins for the production of antitoxins, and found that the antisera so prepared could be effectively employed for the neutralization of all toxins with the exception of the

enterotoxin. An antiserum prepared with a *Staphylococcus* strain, which produced both potent toxin and enterotoxin when added to a filtrate containing enterotoxic substances and injected intraperitoneally into kittens, prevented vomiting, but when the same mixture was injected intracardially into kittens, or intravenously into monkeys, poisoning symptoms occurred. In an attempt to differentiate food poisoning from non-poisoning strains, Kupchik(30) applied various cultural and biochemical tests, but none of the ordinary tests was successful. Intraperitoneal inoculation of kittens gave fairly satisfactory differentiation, since by this means twenty out of twenty-five strains from food poisoning were found to be capable of producing enterotoxins, whereas enterotoxin production could be demonstrated only in nine out of nineteen strains from other sources. When rabbits were given intravenous or intraperitoneal injections with heat-treated filtrates, those containing enterotoxins produced diarrhoea within 1 hr. and death usually within 24 hr.

That outbreaks of staphylococcal food poisoning are not more numerous than they are must be considered rather remarkable, since recent investigations into the etiology of bovine mastitis has shown that numerous cows are infected with staphylococci. Gwatkin *et al.*(31) examined a series of 260 cattle and found 143 affected with mastitis; in thirty of the affected animals the *Staphylococcus* was definitely incriminated, and twenty of the thirty cases of staphylococcal mastitis occurred in two herds in which the *Staphylococcus* was the only pathogenic organism isolated. Minett(32) has recently fully reviewed the literature on this subject, and has added further knowledge. In a series of milk samples from 415 cows from five herds, 145 cows, or 34·9%, were found to be infected; and infection appeared to be more common in the hind-quarters than in the fore ones. In distinguishing pathogenic staphylococci from non-pathogenic types commonly present in normal udder milk, emphasis is laid on the importance of the technique adopted. Plain agar or agar containing horse blood should not be used. Agar containing sheep or ox blood is suitable, especially if the blood is taken from young animals. Further observations made by this worker showed that sixteen out of thirty-eight strains of *Staph. aureus* isolated from the bovine udder were capable of producing enterotoxins. Some of the thirty-eight strains were recovered from cows with acute mastitis, eight from cases of chronic mastitis, and the rest from the normal milk of seventeen cows. In these three groups enterotoxin production was demonstrated with four, five and seven strains respectively. Twelve strains of saprophytic (non-haemolytic) staphylococci from the milk of as many cows were tested, but enterotoxin production was detected with one strain only, and then only once out of three attempts. That mastitis in animals is not confined to bovines is elicited from a paper by Cubeddu(33), who describes a similar condition in goats, and further, Schmidt(34) records illness in human beings who consumed milk from a goat suffering from staphylococcal mastitis. Again, Minett(28) has shown that the enterotoxin can be produced under natural conditions, since the substance can be demonstrated in milk seeded with *Staph. aureus*, or naturally contaminated with that organism and stored at atmospheric temperatures 18 and 22° C., and further, that the substance can remain active in cheese made from such milk.

The prevalence of food poisoning due to the *Staphylococcus* has not been extensively recognized in this country. It is probable that many such outbreaks occur, but as the patients affected are usually only mildly ill the public health authorities are not notified of the occurrence, or are notified only when it is too late to obtain satisfactory specimens for final proof. An outbreak has been described by Scott(35), associated with milk and custard, and another described by Fanning(36) was believed to be due to infected butter. In America, raw milk and products made therefrom, such as ice cream, cheese, cakes filled with custard and cream, account for a high proportion of the recorded outbreaks, and the symptomatology of staphylococcal

food poisoning is well illustrated in a paper by Denison (37). This author described an outbreak due to the consumption of cream cakes in a school of some 693 students; 165 of these ate cream puffs and ninety-four, or 57%, were taken ill. In addition, twenty-eight cases occurred in individuals outside the school who had partaken of the same food stuff. The earliest onset after ingestion was 3 hr., 80% of the cases were ill after 4 hr., and in the remainder the illness was delayed to 6, 7 and 8 hr. The following table summarizes the incidence of the various symptoms:

Symptoms	Information from no. of cases	None %	Mild %	Severe %
Vomiting	122	12·1	9·7	78·2
Pain in abdomen	122	4·7	32·8	62·5
Diarrhoea	103	12·6	72·8	14·6
Headache	101	29·0	58·0	13·0
Muscular cramps	113	36·3	51·3	12·4
Sweating	100	33·0	67·0	—

Again, Geiger (38) recently reported on 110 cases in twenty-eight families affected by staphylococcal food poisoning from eating cream custard cakes.

The prevention of staphylococcal food poisoning from milk or milk products would appear to necessitate heating the milk or cream, together with the additional constituents, to a sufficient temperature to destroy the staphylococci. Dack (39) showed that heating to 80° C. for 15 min., and McBurney (40) to 85° C. for 10 min., was sufficient to destroy all strains tested. Thereafter the material ought to be cooled and stored in refrigerators until required, when again every precaution should be taken to prevent contamination. The addition of a starch to the milk seems to enable staphylococci to elaborate their enterotoxins more freely. Furthermore, Minett (28) notes that strains of staphylococci which were apparently non-enterotoxin producers could have this property restored by culturing on a solid medium containing starch.

III. Bovine tuberculosis

The importance of the various aspects of the problem of human infection with the bovine type of tubercle bacillus cannot be over-emphasized since, from the public health point of view, the continued, rather complacent attitude in this country to an easily preventable disease calls forth expressions of wonderment in other countries.

Several papers dealing with mycobacteria in general, and the tubercle bacilli and their products in particular, require to be mentioned. Long (41) discussed the disease due to the mycobacteria, and concluded that, in addition to those occurring naturally, certain variations could be produced at will: (1) from one strain of mycobacteria in different animal species, (2) from different strains of mycobacteria in one animal species, (3) from one strain of mycobacteria in one animal species modified by immunization, (4) from one strain of mycobacteria in one animal species with genetic variability in susceptibility, and (5) from the dissociated elements of one strain of mycobacteria in one animal species. Gordon (42) studied the cultural reactions of some 252 strains of saprophytic acid-fast organisms obtained from soil plants, human and animal tissues, and secretions. Of these strains 80% were readily separated into three groups of closely related cultures: (1) their ability to withstand heating at 60° C. for 1 hr., (2) their capacity for growth at 47° C., and (3) their power to utilize arabinose and sorbitol. The remaining 20% of the strains which differed from the above, and in most cases from each other, were arbitrarily distributed among the three major groups as subgroups. Wells (43) described a disease in field voles due to acid-fast bacilli. Voles both dead and alive were examined from areas in Scotland, England and Wales. The bacilli produced a fatal disease in guinea-pigs, but a non-fatal one in rabbits, though the lesions produced at the site of inoculation were extensive. Griffith (44) reported experiments on field voles, and showed that these

animals are highly susceptible to bovine tubercle bacilli, and that they can also be infected with human bacilli though apparently with less certainty than with bovine strains. Wells (45) has confirmed the findings of Griffith, and has suggested that a dose of 0·001 mg. of most cultures of tubercle bacilli injected intraperitoneally into voles should distinguish unfailingly between human and bovine tubercle bacilli in one month, all those which received bovine bacilli showing extensive disease, while those which received human bacilli failed to show any macroscopic signs of infection. No evidence has been adduced as to the nature of the organism isolated from the voles, or to what extent it is pathogenic for animals other than the guinea-pig or rabbit.

Parish (46) has contributed a review on the modern outlook on tuberculin. He deals with the preparation and purification of tuberculin, its standardization, its use in diagnosis and epidemiology, in prophylaxis, in treatment, and in veterinary practice. He concludes that the purified protein derivative (P.P.D.) is the best reagent so far produced for diagnostic tests in man, and that the Mantoux test should replace all others in the diagnosis of tuberculous infection, since it permits accurate and graduated dosage. He suggests that so far as human infections are concerned, treatment under controlled conditions might be resumed. As regards veterinary practice, he emphasizes the fact that the intradermal test with potent tuberculin is highly specific in cattle, and is a reliable indication of the presence of infection in cattle, pigs, and fowls.

From the point of view of technique, no new developments can be recorded. Tytler *et al.* (47) have, however, made an exhaustive report on the various methods employed for the demonstration of tubercle bacilli in sputum. They conclude that both cultures and animal inoculation are considerably more sensitive than direct film examination, even when the latter is made frequently. For concentration they advocate the use of the Pottenger (48) technique as modified by Edwards *et al.* (49), and called by them the Gasolene Concentration Test. It has long been accepted that guinea-pig inoculation was the most sensitive method for the demonstration of tubercle bacilli, but the authors point out that there are now on record a number of investigations in which the cultural method has been found to be slightly more successful than guinea-pig inoculation. The findings indicate that for the human type of tubercle bacillus, culture was more successful for all types of specimens except urine. For bovine bacilli, which are more difficult to isolate on artificial media, and are somewhat more virulent for the guinea-pig than the human type, the results of culture and animal inoculation are practically equal for all specimens except urine, in which the inoculation method is again superior.

The incidence of human, non-pulmonary infections with the bovine type of tubercle bacilli has again been the subject of further investigation and discussion. Griffith (50) dealt at length with the problem of bovine tuberculosis in man, and gave the complete statistics for the incidence of bovine infections in non-pulmonary tuberculosis for England and Scotland. Lange (51) has summarized the position in Germany, and also in other continental countries. The following table has been compiled from the papers of the preceding authors, and that of Jensen (52):

Percentage incidence of bovine tuberculosis in cases of non-pulmonary tuberculosis

Type of tuberculosis	England Griffith	Scotland Griffith	Germany Lange	Denmark Jensen
Cervical adenitis	50	51·6	22·3	49·0
Bone and joint	19·5	29·8	4·6	18·5
Genito-urinary	17·4	31·0	0	—
Lupus	48·7	69·2	24·3	—
Meningitis	24·6	29·2	0	24·7
Autopsies	22·5	32·4	9·4	—
Miscellaneous	8·7	71·4	—	—
Abdominal	—	—	32·7	—

Owing largely to the outstanding efforts of Griffith, the statistics for England and Scotland are very complete, and the number of cases now examined must be sufficiently large to be of real value. The typing of tubercle bacilli in Germany and Denmark has also been carried on fairly extensively, but in other countries the number of specimens examined from different lesions has not been large. For full details of the various investigations the reader is referred to the paper of Lange (51) Further papers dealing with the incidence of human tuberculosis of bovine origin are those of Dalrymple-Champneys (53) and Morgan (54). The first paper deals with the incidence in Great Britain generally, and the second with the incidence in Scotland in particular.

Fresh interest has recently been aroused in this country and abroad in the incidence of pulmonary tuberculosis due to the bovine type of bacillus. Koch in 1908 had declared that in no case of chronic pulmonary tuberculosis had the bovine bacillus been found. Griffith had, however, proved its occurrence in three cases up to the end of 1913. Even until 1922 only four persons, two in England and two in Scotland, had been found to have bovine tubercle bacilli in the sputum, when an investigation by Munro showed that two out of 100 strains isolated from sputum were bovine tubercle bacilli. The findings to date have been summarized by Griffith (50) in the following table, in which the figures for the north-east of Scotland are amended to conform with those published more recently by Griffith & Smith (55):

Proportional frequency of human and bovine types of tubercle bacilli in sputum

Country	Region	Authors	No. examined	Human	Bovine	% bovine
Scotland	North-east: Rural	Griffith & Smith	342	311	31	9·1
	North-east: City	Griffith & Smith	294	281	13	4·4
	Middle and south	Munro	947	902	45	
		Griffith	495	474	21	4·6
		Wang	29	28	1	
England	North	Cumming	888	874	14	1·6
	Middle	Lynn & Cuthill	803	791	12	1·5
	Middle	Griffith & Menton	230	228	2	0·9
	South	Griffith	690	686	4	0·6
	South	Cumming	195	194	1	0·52
Wales		Cumming	203	201	2	1·0
		Selected cases				
	Various in England	Cumming	245	224	21	
				Total bovine	167	

In the figures given by Griffith & Smith (55), the incidence of bovine pulmonary tuberculosis in patients in the rural area of the north-east of Scotland is more than twice that in city cases. This is no doubt due to the fact that in the city of Aberdeen at least 80% of the milk is effectively pasteurized, whereas in the country areas the milk is supplied entirely in the raw form. In Holland, Ruys (56) found bovine tubercle bacilli in the sputum of three of 115 adults in Amsterdam, and of ten of eighty-nine adults in north Holland, giving a percentage of 6·4 for the total of 204 cases. In Germany, Lange (51) reported that he had examined sputa from 148 adults, and found the bovine bacillus in nine cases, and both bovine and human types in three. Lange also calculated that of the 50,000 persons who die of tuberculosis every year in Germany, 1000 die of the bovine infection. This author also emphasized the importance of contact with cattle, for example, workers employed in dairy farms, as the explanation for infection with the bovine type of bacillus. The findings of Griffith & Smith (55), however, point rather to the consumption of raw, infected milk as the chief source.

Actual epidemics of tuberculosis are of comparatively rare occurrence, but several such have been described recently. At the present time erythema nodosum is believed

by some to be a manifestation of rheumatic disease rather than a tuberculous infection, but the experience of Swedish observers points definitely to its association with tuberculosis. Törnell(57), however, who had previously observed three epidemics of erythema nodosum which were believed by him to be due to tubercle bacilli of bovine origin, described a fourth which occurred in the summer of 1935, involving thirteen women factory hands, whose ages ranged from 17 to 29 years. All the patients were tuberculin-positive, and with only two exceptions the radiological examination of the lungs was negative. The only common food factor was the milk supply, and this was found to contain tubercle bacilli. Again, Stahl(58) reports an epidemic in a Swedish community in which fifty persons, mostly children, showed signs of a tuberculous infection. In addition, 166 persons, of whom 134 were school children, gave a positive tuberculin test. Enquiry showed that there was a common milk supply, and one cow in the herd was found to be suffering from tuberculous mastitis. Of the fifty persons infected, some showed high fever which continued for several weeks, others enlargement of the cervical glands, and some erythema nodosum.

In a report of the Cattle Diseases Committee of the Economic Advisory Council, an estimate was made for the year 1931 that in Great Britain there were at least 2600 deaths and 6000 new cases caused by the bovine type of tubercle bacillus. The need for effective control is therefore undoubted. That this can be obtained by pasteurization has been shown by Humphriss *et al.*(59). These workers examined 440 samples of "school milk" treated in commercial plants as required for pasteurization. None contained tubercle bacilli. From one plant alone 102 samples all gave negative results, although no less than sixty-one of the corresponding raw samples showed the presence of tubercle bacilli. The results of the experiments indicated that, under ordinary commercial conditions, properly run and adequately supervised pasteurization is a completely effective process.

IV. *Salmonella* Infections

The *Salmonella* infections can be divided into two categories, namely, those infections with a typhoid-like symptomatology, and those with the characteristics of food poisoning.

At the present time the Vi, H and O antigens of the typhoid bacillus continue to be the subject of considerable investigation, particularly in regard to their utilization in the diagnosis of typhoid fever, and in the preparation of therapeutic sera. Felix & Pitt(60, 61), Felix & Bhatanagar(62), and Bhatanagar(63) have shown that (1) antibodies to the Vi antigen are produced in every case of typhoid fever, (2) that Vi agglutination is shown to be a more reliable method of diagnosing typhoid fever in the inoculated than the H and O method, and (3) that the detection of Vi agglutinins in the serum of suspected carriers is of great value since the presence of these antibodies is practically indicative of a carrier condition. As regards epidemics, the *U.S. Public Health Service Reports* summarize the milk-borne outbreaks as follows:

U.S.A. milk-borne typhoid and paratyphoid

	1936			1937		
	Epidemics	Cases	Deaths	Epidemics	Cases	Deaths
Typhoid	15	95	5	15	161	11
Paratyphoid	1	21	0	—	—	—

In this country no epidemics of importance associated with milk have occurred, but the full report on the large outbreak which occurred at Bournemouth and adjoining towns in 1936 has now been contributed by Shaw(64).

As regards the food-poisoning members of the *Salmonella* group, several additions have been made to the already numerous types. Kauffmann (65) discussed the present position of the problem of identification, classification, and nomenclature, and gave detailed tables of their antigenic structure. Investigation into the incidence of various types of food poisoning organisms in animal specimens has been made by Khalil (66) in rats, and Scott (67) in healthy pigs. The incidence of *Salmonella* types in bovines has not been intensively investigated. Knoth (68) reported that he had isolated 561 strains of *S. enteritidis* in animals slaughtered in Leipzig between the years 1930–5, but he did not, unfortunately, carry out any serological subtyping. Lesions such as metritis, mastitis, and gastro-enteritis were noted in the affected animals.

One type of *Salmonella* is becoming more frequently associated with milk infections, namely, the Dublin variety. A report by Conybeare & Thornton (69) describes an outbreak of food poisoning involving over 100 persons in Wiltshire. The outbreak was due to bottled raw milk, and the source of infection was traced to a single cow apparently healthy, but from whose dung the organism was repeatedly isolated. Again, Pullinger & Kemp (70) have investigated the sequence of events which follow when various Salmonellas are introduced into milk held under ordinary conditions of commercial and household storage. *S. typhi* and *paratyphi* and various other members of the *Salmonella* group were found to multiply readily in raw and heat-treated milk when stored at 15° C. When incorporated in various grades of salted and unsalted butter, these organisms were found to survive for at least two months.

V. Dysentery

At the present time four main groups of dysentery bacilli are recognized: *B. dysenteriae* Shiga, Flexner, Sonne, and Newcastle. So far as this country is concerned, infections with the Shiga type are relatively uncommon, whereas infections with the other three varieties are of frequent occurrence, and the disease reached epidemic proportions during the past year. The disease is not usually spread by the contamination of water or milk, but by actual transmission of infected material from one individual to another. Occasionally, however, a milk supply becomes contaminated, with the result that a sharp, explosive outbreak of the disease occurs.

During the past two years only two epidemics of dysentery are recorded in the literature as being due to infected milk supply, while another outbreak was associated with a milk product—ice cream. The first occurred amongst (Heanley (71)) young European children in Hong Kong, who were consuming unpasteurized milk. In all, twenty-three individuals were affected, of whom seven died. The organism isolated from this outbreak was the Shiga bacillus. The second outbreak, described in detail by Bowes (72), was of somewhat larger dimensions. Over a period of 5 days, ninety-six individuals in the town of Bedford were affected with symptoms of mild enteritis. The symptoms consisted of abdominal pain, vomiting, and diarrhoea for 12–36 hr. with, in a few cases, blood and mucus in the stools. Inquiries by the Health Department showed that all cases had received their milk supply from one retailer in the town. The milk had been distributed to 106 households, and in fifty-nine one or more members had been affected. From various specimens of faeces, and from a sample of milk taken some four days after the onset in the first cases, *B. dysenteriae* Sonne was recovered by Dr Scott of the Ministry of Health. The milk supply was unpasteurized, and, so far as could be ascertained, the infection could not have occurred at the farm which supplied the milk but must have occurred at the premises of the retailer.

Savage (73) recorded an extensive outbreak of Sonne dysentery due to ice cream. The outbreak was scattered in nature, and involved no less than 114 individuals in five different sanitary areas. The affected ice cream was distributed by an itinerant

vendor who, with the aid of certain members of his family, prepared the material. The incubation period varied from 24 hr. to 2 days, the symptoms were mild in character, and the patients affected were mostly children. The bacteriological investigation failed to reveal the actual source of infection.

VI. *Brucella* infections

In a series of papers Evans (74, 75), Poston (76), and Evans *et al.* (77) have examined the various laboratory tests as applied to the study of chronic brucellosis in man. These workers comment on the protean manifestations of the disease and its occurrence in atypical, chronic and latent forms. They have described the technique and evaluated the results of the various laboratory procedures which would be helpful in establishing a diagnosis. They believe that no single test can be relied on to determine *Brucella* infection in a given case. They found (1) that an agglutinin titre of 1 in 40 or higher occurred about eleven times as frequently in chronic brucellosis as in cases ill with other diseases, (2) that a positive skin reaction occurred four times as frequently in chronic cases of the disease than in other infections, and (3) that the opsono-cytophagic reaction was found to be the least reliable of the specific tests, because positive reactions are not uncommon in cases ill with other diseases. The three tests, agglutination, skin and opsono-cytophagic, when positive, occurred about ten times as frequently in chronic brucellosis as in cases ill with other diseases. Finally, two cases from whom *Br. abortus* had been obtained gave negative immunological tests. Taylor *et al.* (78) also give a very complete description of the technique of the various methods, bacteriological and serological, which they have employed in the investigation of undulant fever in France. In a series of 1412 human blood specimens, they have compared the agglutinin titre of the serum with the results of blood culture. They found that the percentage of positive blood cultures increases with the rise in agglutinins until a titre of 1/160 is reached, after which there is no significant difference. The high percentage of successful blood cultures was probably, to some extent, due to the fact that some 95% of the strains isolated belonged to the *melitensis* variety. They discuss in detail the possible explanation of the failure to obtain a positive agglutination test in a dilution of 1/80 or more in thirty-nine instances in which the blood culture was positive.

In sheep and goats, 18 and 15% respectively of those which gave a positive agglutination test gave positive blood cultures. In sheep, goats and guinea-pigs the sheep allergic reaction was found to correlate with the agglutination test to a high degree, although the latter missed a few more animals, proved later to be infected, while it also gave a slight excess of false positives.

The incidence of known cases in different countries varies considerably; thus the reports of the U.S. Public Health Service and the Chief Medical Officer for England give:

	England		U.S.A.	
	Cases	Deaths	Cases	Deaths
1935	391	—	2008	98
1936	48	—	2095	107
1937	—	—	2497	58

Dalrymple-Champneys (79) discussed the prevalence of undulant fever, and believes that Wilson's (80) estimate of 400–500 cases per annum in England is not an exaggeration. He commented on the diagnosis, avenues of infection and measures of control in this disease. He pointed out that Section 5 of the Milk and Dairies (Consolidation) Act, 1915, makes it an offence to sell, or offer, or expose for sale for human consumption the milk of any cow suffering from certain diseases, and the Milk and Dairies

Order, 1926 (Section 1), lays down that the diseases specified in the Act shall include "any infection of the udder or teats which is likely to convey disease". The Ministry of Health holds the view that the discovery of *Br. abortus* in milk is evidence of the existence of such a condition, and consequently Section 5 of the Act would apply to a cow giving such milk.

Hagan (81) has reviewed existing information on the animal reservoirs of organisms of the *Brucella* group. He concludes that it has been adequately proved that human infections occur as a result of contact with cattle, goats and swine, and their secretions. There is also little doubt that horses act as sources of infection, but he believes it is highly improbable that sheep, dogs and fowls, even though they may be invaded by *Brucella*, ever transmit infection to human beings.

Taylor *et al.* (78) found the *abortus* type to be relatively unimportant in southern France as a cause of undulant fever in man, most cases being due to *Br. melitensis*. The disease tends to show a marked seasonal variation, reaching a peak in spring corresponding to the period of abortion and parturition in domestic animals. Relatively few human cases occurred in individuals under 15 years, and the disease was two to three times more frequent in males than in females. Goats, sheep and cows constitute the animal reservoirs, and the cow is not infrequently infected with the *melitensis* variety. Their studies pointed to the fact that the risk of infection through contact with animals is definitely greater than through consumption of raw milk or cheese, and that sanitary conditions and environment are of limited significance.

Much work continues to be done in connexion with the eradication of contagious abortion in cattle, and progress is being maintained. The solution to the problem of human infections in this country would be either eradication of infection in dairy herds, or the pasteurization of the milk supply. The disease, though crippling to the individual who is unfortunate enough to contract it, is not a problem of major public health importance.

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SECTION G. GENETICS OF DAIRY CATTLE

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Milk yield
Shape of the lactation curve
Sex linkage
Butterfat
Protein
Other constituents
Colour and flavour
Vitamin content
Curd tension
Conformation
Efficiency of production and growth
The progeny test
Reproduction and wastage
Breed and herd construction—inbreeding
Tropical
Artificial insemination

Milk yield

So long as any studies are made upon the inheritance of milking capacity, so long will there be debate as to the relative influence of heredity and environment. In past reviews on this subject, emphasis has been laid upon the work of Von Patow, who produced the "byre average" as a means for assessing the true genetic worth of dairy cattle. In the last review a paper by Lush was quoted, treating the "byre average" from a purely statistical angle, in which he showed that the method was unlikely to lead to a Mendelian analysis which corresponded to reality. The outstanding paper which now falls for review is by Lörtscher(1), a Swiss investigator, who has worked in Edinburgh at the Institute of Animal Genetics, under von Patow at Berlin, and with Dr Schmidt at Zurich. Some 4000 lactation records of 1200 cows from a group of British Friesian herds (Terling-Lavenham) in England provide the data. The yields of the individual cows, reduced to the "byre average" by the von Patow formula, were shown to normalize the frequency of distribution, as predicted by Lush. The figures obtained were found to fit von Patow's genetic hypothesis of milk inheritance. Out of 833 daughter-dam comparisons, in only 0·6% was the genotype of the one not in agreement with that of the other. The genotype of the bulls was ascertained by comparing the genotypes of the daughters to their dams. Of forty-three bulls with at least five tested daughters, all were heterozygous for the three factors belonging to those genetic classes which allow the largest possible combinations of genes when mated to cows of all classes. With the bulls arranged according to the periods they were in service in the herds, the distribution of their daughters, with respect to the seven genetic classes, does not show any increasing asymmetry, and for only two of the forty-three bulls was there a small, but significant, difference between the mean of the daughters and of their dams. Accordingly, the author is forced to the deduction that, according to von Patow's hypothesis and method of investigation, there has been in these herds no success as a result of selective breeding for high milk production during the past 20 years. Employing Krüger's adaptation of the "byre average" (where the yields are corrected for certain environmental factors before being reduced to the average) no significant difference was

found between it and von Patow's method. A similar conclusion was reached from a study of the females, that no significant increase in milk yield was due to breeding.

Nevertheless, the average yield of the cows in the herds was increased from 700 gal. in 1910 to over 1100 gal. in 1932. As this conclusion does not square with experience, the question therefore arises whether the results obtained were caused by a systematic error. Accordingly, a second method of investigation was pursued based on the amount of inbreeding and the relationship between groups of cows born in the same year, with an interval of 4 years from group to group. Sewall Wright's coefficients were employed. As constructive inbreeding occurred in these herds excellent data were available for a study of this nature. The figures obtained lead to the assumption that, in the course of time, the effect of constructive breeding and selection for high milk production would be of such a measurable degree that it could not be counterbalanced or elaborated by environmental influences. This contradictory result is explained as due to the reduction of the yearly records on the "byre average", which supposes that the variation between years is of purely environmental origin, whereas, in fact, the differences from year to year in the "byre average" are partly due to the working of genetic factors.

A further analysis was made by an examination of (*a*) the influence of varying average age, average length of dry period and of service period upon the "byre average"; (*b*) the influence of selection and breeding, i.e. of the genetical composition of the herd in consecutive years; (*c*) the relation between single yield and "byre average" by varying environmental conditions to examine whether a linear equation may be used to reduce the yields on the "byre average". To avoid the wearisome work connected with (*a*), of multiplying each yearly record with three corresponding correction factors, a nomogramme was constructed, which proved exceedingly useful, and is recommended for similar investigations.

The variance within each year showed three typical periods, 1910–16, 1916–22 and 1922–30. Within these periods no significant difference was obtained between the variance within years; but a comparison of the variance of years belonging to different periods showed significant differences, especially when the years prior to 1916 were compared to those subsequent to 1922, which is attributed to the effect of line-breeding. By estimating the influence of purely environmental conditions on the difference between the means of two and two successive years, an estimate was made of the effect of selection and of the change in the genetical composition of the herd. (Details are given of the method employed according to a formula suggested by R. A. Fisher.) The differences are statistically significant, and the conclusion is confirmed that, by the reduction of yields on the "byre average", part of the genetical variation is eliminated.

By subtracting from the total variance the portion caused by environment, the latter being obtained by halving the variance of differences between yields of the same cows for two and two successive years, a further estimate of genetic variation was obtained, from which the conclusion is drawn that from 50 to 70% of the variation is due to genetical causes. The author states that this method is not entirely accurate, since many cows are in the herd for longer than two successive years, and the formula does not allow for repeated appearance of the same animals.

Studying the relation between single yield and "byre average" by changing environmental conditions, Lörtscher compared the variation coefficients of the yearly records of the same cows in two successive years. The use of a linear equation to reduce the yields in the "byre average" includes the assumption that a change of environmental conditions affects all yields proportionately. If this were true, the variation within each year would change in the same way, and to an extent proportionate to the change of the "byre average" caused by environmental conditions

only. Thus the variation coefficient of the yields of the same cows in successive years should remain constant. In none of the comparisons was this the case. Accordingly, it is concluded that an increase or decrease of the "byre average" does not change the variation within years proportionately in the same sense; each individual cow appears to have a different power of reaction against varying environmental conditions according to its genotype. Thus, if the "byre average" is to be used, it must first be corrected for the effect of changing genetical composition; thus its use defeats its own object.

The assumption of a working hypothesis as regards the inheritance of milk yield (or any other productive character) is useful for practical purposes, but in purely scientific investigations such deductions, based on the principle of trial and error, have the disadvantage that, in the case of a failure, there is no evidence as to the probable mode of inheritance. Lörtscher concludes that, for characters such as milk yield, which are much affected by environmental conditions, it is exceedingly difficult to obtain numerical values which truly represent the genotype of single individuals. The elimination of varying environment by the use of correction factors only partly overcomes the difficulty, since such characters are corrected only for the average of many and seldom for the single individual.

Dealing with specific environmental factors which affect the yield, Lörtscher obtained significant positive correlations in the case of yield and age of cow (+0·169), yield and length of lactation (+0·535) and yield and length of service period (+0·461). As regards dry period, a positive correlation was obtained between the age of the cow and the length of the dry period preceding the lactation record, showing that older cows are usually given a longer rest before calving. The shortest dry periods fall between April and June. A dry period should never be less than 3 weeks, while, to obtain a maximum yield in the subsequent lactation, it is advisable that the dry period should be from 8 to 10 weeks; a dry period longer than ten weeks results in a lower lactation yield, probably as a result of fatty degeneration of the udder. Once allowance was made for the fact that most of the heifers tended to calve in autumn, month of calving was found to have no influence upon lactation yield, a tribute to the management of the herd. The bearing of twins had no effect upon the lactation yield, but it is noted that gestation with a female foetus shows a somewhat shorter dry period than when the cow is pregnant with a male.

Of computed partial correlations, only two proved significant, yield/age of cow and yield/length of lactation. As regards age, the maximum of the curve corresponds to 7–8 years, while the curve for length of lactation shows that an increase of the number of days in-milk does not raise the yield proportionately. Generally speaking, the results show that between some of the environmental factors there exist interrelations; to avoid over-correction, these must be considered when correction factors are calculated.

This subject is briefly reviewed by Turner (2). Pak (3), analysing 247 lactations of sixty-one cows of the Dutch breed, showed a correlation between length of lactation and yield during 180 days amounting to 0·130 for all lactations. A small, but significant, correlation was found between month of calving and yield. Cows calving first at 3 years of age yielded a first lactation record of about 60%, and a second of about 70–80% more than those which calved first at 2–3 years. Length of dry period was found to have little effect. Correlations are also given concerning the accuracy of yields over a short period as an indication of the total lactation. Chapman & Dickerson (4), with 253 American Holstein cows, found an irregular tendency for yield to increase with increased age at first calving. They noted that, at 7 years, early calvers had produced considerably more butterfat, and approximately one more calf than late calvers. The mature weight of the animal was not impaired. Kleinberg (5),

dealing principally with weight, finds slight increases of milk and fat production with age: the length of lactation was independent of age and correlations between milk production, fat yield and fat content were independent of age and weight.

Krüger (6), studying the average discrepancy between a cow's uncorrected record and her real producing ability, comes to the conclusion that it is about 20% of the record in the data from Cow Testing Associations in Silesia. He states that the use of correction factors for age, service period, lactation length, etc., is helpful, but is never complete. He outlines the application of this method as an improvement on von Patow's "byre average" (see observations of Lörtscher above). Scherler (7) applies Krüger's factors to four herds of German cows. From the corrected data he claims that it is possible to compute values for the hereditary capacities of individual cows and bulls, using the method of the heredity grid. He concludes that it would be hazardous to draw general inferences on the breeding values of individuals and that conclusions should be restricted to families tested. The data proved insufficient for testing von Patow's hypothesis of the inheritance of milk yield. He finds the best age for first calving to be from $2\frac{3}{4}$ to 3 years. Bode (8), also for German cows, concludes that if they are less than 3 years of age at first calving, their subsequent milk production is adversely affected. It is interesting to compare these with the recognized figure of 30 months for British and American cows and 24 months for the Channel Island breeds. Johansson (9), in a survey of modern problems of animal breeding, believes that the experimental method for a study of the inheritance of milking capacity can never be free of great sources of error, and accordingly prefers statistical investigations based on large numbers. Referring to the "byre average", he accepts it only as a rough approximation, and prefers to believe that both yield and quality of milk are conditioned by a large number of genes. Lahaye & Cuperstein (10) recommend as a basis for selection the comparison of the daily yield of a cow with the "byre average" during the appropriate period of its lactation plus a preparatory period of 90 days preceding calving. On applying this method to a herd of 21 cows, it was found that the results differed considerably from the figures of total yield. Abts (11) finds the value of the "byre average" to be problematical.

Regan & Richardson (12), dealing with the effect of environmental temperature, conclude that, as the breathing of a cow varies directly with environmental temperature, her skin has a high insulating value, and that she may therefore adapt herself with comfort to conditions of extreme cold. This explains why exposure to zero weather brings about neither lowered production nor increased maintenance requirement. Butterfat secreted when the cows were kept at a higher temperature was lower in volatile acids and higher in unsaturated components, while the *p*H content of the milk was raised and the freezing-point depressed. Swett *et al.* (13) show that the loss of a quarter in the udder of a cow does not necessarily diminish her yield in proportion: the ability of nature to compensate for such a loss is stressed.

Shape of the lactation curve

A paper on this subject by Nielsen (14), dealing with Red-and-White Swedish cattle and comprising 193 lactations, with milk production standardized to 4% fat, shows that in one group, composed on the average of rather older cows, there were only six which showed large deviations from the general regression coefficients described by Bonnier. He describes a "point system" for measuring the shape of the lactation curve. Gilmore *et al.* (15), with records from the University of Minnesota and from Cow Testing Associations, show that the hereditary factors which affect the initial yield of a cow at the commencement of lactation are different from those which affect the persistency of lactation. Copeland (16) discusses the difficulty of evolving

a single numerical figure with which to estimate the persistency of the lactation curve. To avoid this, he selected groups of cows with records extending to 365 days. The first group had completed records of over 1000 lb. of butterfat, the second were definitely low record cows, while the third consisted of cows calving with the same interval, but with shorter lactations. For each cow, records were divided into twelve 30-day periods. The results show that the total production for a lactation period gives some indication regarding persistency, since the high-producing cows were, in the majority of cases, more persistent than the extremely low producers. The high initial yield of milk or butterfat gives little information regarding persistency, since cows with the same initial yield varied greatly. The author emphasizes how the persistency characteristic of the lactation curve persists throughout the lifetime of a cow, and emphasizes the genetic aspect of the question. Gooch (17), working also with Jerseys, describes the trend in the amount of milk produced by a single exponential curve. A negative correlation was found between persistency and high initial yield, but neither were correlated with the scatter of the observed monthly yields. He shows that, while there has been some selection for high yield, based probably on high initial yield, there has been no apparent selection for persistency. Total yield shows a slight negative correlation with both persistency and variability of monthly yields. Time of calving had a slight effect upon persistency, the more persistent records being from cows calving in the winter months, while those calving in early spring had a higher initial yield. A dry period of more than 8 weeks before a given lactation is associated with increased initial yield, decreased persistency, and greater variability in monthly yield, while milking for a longer time has the opposite effect on each of the three characters (see Lörtscher above). In both initial yield and persistency, variation between lactations of the same cow is greater than between different cows.

The late Dr Kronacher & von Patow (18) analysed the records of the dairy herds at the Dahlem Experimental Stations. Examining the form of the lactation curves of twenty-six cows (forty-four lactations) no criterion was obtained on which predictions as to future performance could be based. No regular influence of age could be detected, but there was a relatively high correlation between total lactation yield and highest daily yield. They also note that the period from service to the point at which the new pregnancy begins to affect the milk yield varies from 107 to 206 days, with an average of 145 days. This period seems to increase with age.

Johansson (19), studying the production records in a herd of Swedish Red-and-White cattle, finds a maximum production at 7–10 years of age, yield unaffected by month of calving, and a higher average daily yield for lactation periods of from 331 to 420 days. He confirms that a short dry period has a depressing effect. The shape of the lactation curve, he states, is influenced by age of the cow, length of calving interval, and, in some cases, by the season of calving. For the period 1921–30 the persistency of yield was greater for autumn and winter calvers than for spring and summer ones, but from 1931 to 1936, when nutrition was adequate throughout the year, the effect of month of calving was negligible. He suggests a numerical index to indicate the shape of the lactation curve in periods not exceeding 300 days. Variations are found between individuals and families in regard to the shape of the curve, and 28% of the variance in the lactation curve was found to be due to variation between animals, whereas the rest was due to variation from year to year. Abts (11) emphasizes persistency of high fat percentage. In conclusion, these studies on the shape of the lactation curve must lead to certain confusion. The subject requires further examination and the answer will probably be found in an extension of the argument as to the relative effects of environment and heredity.

Sex linkage

Buchanan Smith (20) reports a statistical enquiry into the inheritance of milk yield in three herds of Dairy Shorthorn cattle. To reduce environmental effect, the study was restricted to each herd considered as a unit, and Fisher's squared difference method was used. The lumped results confirm previous work, to the effect that the paternal grandsire has a lesser effect than the maternal grandsire, but, in this respect, the figures for one herd did not agree with the other two. On the largest herd (which showed this difference) being divided into two sections of high and low yielders, each section did not show any difference in the relative effect of the two grandsires. The implications of this are discussed, and it is tentatively suggested that, if sex-linkage operates, it has a considerable effect of some 250 gal. on an average yield of some 600 gal. The author concludes that the accuracy of the conclusions that may be drawn from statistical studies of this type are not commensurate with the labour involved (thus disagreeing with Johansson above). As a reconnaissance, such studies have definite value, but planned experimentation is required to resolve the problem. Lörtscher (1), with the material described above, and using Fisher's method of dividing the squared daughter-dam correlation by the daughter-grandam correlation, calculated the correlations between daughters and grandams, finding a slightly higher figure to the paternal grandam, which might be attributed to sex-linked inheritance. This author is rightly cautious as to drawing conclusions on this point since, with the material used, the higher correlation to the paternal grandam might be attributed to line-breeding. The difference between the daughter-paternal grandam correlation (+0·154) and the daughter-maternal grandam correlation (+0·139) is not significant. Gilmore *et al.* (15) found that differences in the initial maximum yield of a group of cows by one sire could be attributed to the maternal grandsire though their persistency was similar.

Leroy (21) enunciates various laws concerning the inheritance of milking capacity in dairy cattle, the second being that sex-linkage does not affect milking capacity. He admits that the hypothesis of von Patow is fragile, but thinks that critics have been unduly severe and that it might be of service as a working hypothesis.

Butterfat

Curiously little work has been done on the question of butterfat during the past two years. The most important contribution to the subject is a paper by Fairfield Smith (22), primarily designed for the plant geneticist, which discusses the inheritance of quantitative characters masked by non-heritable variations which make it difficult to determine the genotypic values of individuals. He emphasizes the need for a "discriminant function" of the observable characters which may best indicate "genetic value". He works this idea out first in theory and then gives an example in wheat. It is possible to express the value of a plant as a linear function of its characters, and, from Fisher's concept of "discriminant functions", it is possible to derive that the linear function of observable characters will be the best available guide to the genetical value of each line. Thus the expectation of "genetic advance" over the mean of the unselected population for any given selection intensity may be estimated and used for comparison of relative efficiencies of various breeding methods. For the dairy-cattle breeder, the most interesting section of the paper deals with the use of ratios as selective factors. The writer shows that the ratio of two characters can never supply more information than can be given by a consideration of both characters separately, and that, as a rule, the information of a ratio is very much less than when each character is considered separately. The deduction is therefore made that the ratio should never be used as the basis for methods of the improvement of

plants. This deduction can equally well be applied to animals, and justifies the present tendency to desist from tracing the inheritance of percentage butterfat in dairy cattle. It is obviously sounder to concentrate upon the two components, total yield of milk and total yield of fat.

An interesting paper on the inheritance of butterfat comes from Lush & Schultz (23) which includes a study of the correlations to ancestors: this paper is dealt with below under "Progeny Testing". Oxley (24) gives the mean and standard error of milk yield and butterfat for the first five tests at 6-weekly intervals of the lactation period: correlation between milk yield and fat percentage amounts to $-0{\cdot}151$ and $+0{\cdot}154$ for morning and evening milkings respectively: he finds a distinct lag between the times of highest milk and of highest fat yields. Bosma (25), with two breeds of Dutch cattle, obtains correlations between yield of milk and fat content amounting to $-0{\cdot}27$ and $0{\cdot}17$. Dinkhauser (26) maintains that increased fat percentage can only be obtained by selection for this character and not by selection for total yield of milk: he emphasizes the necessity of using proven sires with high butterfat content. Copeland (27), with American Jerseys, obtained correlations between dam and daughter as regards fat yield of $+0{\cdot}29 \pm 0{\cdot}047$, calculated on their highest (age-corrected) records as against $+0{\cdot}30 \pm 0{\cdot}046$ as calculated on their average records: from this and other extremely interesting data he concludes that the highest record of a cow gives about as much information of her transmitting ability for fat production as does the average of a series of records completed during an entire lifetime. This observation is of fundamental importance and this paper deserves close examination.

Emphasis continues to be laid on the question whether butterfat content or yield can be affected by nutrition. Turner (2) provides new data on breed variation in quantity and butterfat yield of cows of the different breeds in the United States, showing the individual variation in the percentage of fat for the four principal breeds. While fat in milk may be formed from other constituents of the feed, he believes it desirable to provide sufficient fat to equal the fat in the milk, and recommends the feeding of 5–6% fat in the grain ration. Two fish oils have a depressing effect on the fat content, while vegetable oils usually cause a temporary increase in the test, but with continued oil feeding the test returns to the normal level. The feeding of butterfat increases the fat content of the milk for a considerable period, but this is hardly economic. A general reduction of the ration causes an increase in percentage fat, due to a lowering of milk yield, though the total yield of fat is also somewhat reduced. Dealing with experiments at Minnesota, Allen (28) reports that the addition of a number of common animal and vegetable fats to the diet results in an increase in the fat content of the milk. Using butterfat, lard, tallow, linseed, cottonseed, soybean, corn, peanut and cocoanut oils, the results were so clear-cut that it was possible to determine accurately by milking at frequent intervals almost exactly the time required after the fat was fed for its influence to appear in the milk. It is claimed that it was possible to secure as much as 20% increase in butterfat percentage by increasing the daily fat intake by 1 lb. or more. No attempt was made to determine whether the higher fat yields could be maintained indefinitely. There was no indication of a return to the normal level after periods of as long as 6 weeks on the higher level of fat intake. He holds that recent research indicates that highly unsaturated fatty acids occupy an important place in the diet; that under ordinary conditions milk fat is deficient in fatty acids with two or more double bonds, and concludes that, for a special grade of milk, it is not inconceivable that the type of ration might be considered in improving the nutritive properties of the milk fat in this respect. Hydrogenated cod liver oil fed to dairy cows had no effect on the fat percentage of the milk according to McCay *et al.* (29), whereas the normal oil fed at $\frac{1}{3}$ ml./kg. body weight lowered the percentage and raised the iodine value. The feeding of desiccated

thyroid has been found by Herman, Graham & Turner (30) to increase the yield of both milk and fat in cows in declining lactation. It also caused a slight increase in fat yield (but not milk) when administered at the peak of lactation. Solids-not-fat were also increased slightly. Thyroxine subcutaneously injected was found to be more effective than the equivalent thyroid given orally.

Watson & Ferguson (31) found no difference in the butterfat yields or butterfat percentage in four different treatments of feeding, which included silage and artificially dried grass. Golding & Burr (32) obtained a slight indication that cacao shell increases the quantity of fat secreted in the milk. Dutta *et al.* (33) fed groundnut and sesame oils to Indian cattle, in addition to the ordinary dairy ration, without altering milk yield or butterfat content; they note the alteration in the physical and chemical composition of the butter according to the particular oil fed. Bratton *et al.* (34) from Purdue report that, with raw soya beans, which were fed in amounts equal to 25% of the grain ration, there was a measurable increase in the fat test, with a noticeably different composition of the resulting fat. The late Dr McCandlish (35) reviewed the subject, referring to work noted in the previous review: he found that specific feeds cannot increase the fat content of the milk, and believed that, in selection for butterfat, breed comparisons are of less importance than individual genetic constitution.

Washburn (36) shows that starvation causes a decrease in milk yield of 50%, and an increase in fat percentage of 100%. The late Dr Kronacher & von Patow (18) reported that individual cows of upland breeds showed, under improved treatment, considerable increase in the fat content of their milk. Johansson (37) reports that it is not possible to increase the average fat content of the milk by thorough stripping. Incomplete milking may cause a decrease in the total yield of milk and butterfat due to the retarding influence on milk secretion of the increased udder pressure, but, generally speaking, fat left in the udder is not reabsorbed, and can be obtained at a subsequent complete milking. On the other hand, Woodward *et al.* (38) find that the milk production of cows incompletely milked amounts to 96·7% of the production when milked completely: incomplete milking has no effect on the percentage of butterfat during the entire lactation period nor does it hasten the decline in milk flow.

Jack & Dahle (39) report that fat globules are electrically charged, and describe the method used for studying the electrokinetic potential of milk fat. There was no significant difference in the mobility of fat globules from cows of different breeds. Hardness of butterfat is usually associated with a low iodine value, and feeds with poor iodine cause a harder fat: this character is also conditioned by heredity according to the work of Hill & Palmer (40) who confirm a previous investigation that breed exercises a definite influence on the hardness of butter.

Protein

Considerable attention has been directed to this subject. McDowall (41), in a substantial paper dealing with the cheese-yielding capacity of milk, states that there exists a breed difference in the casein content of the milk of different cows, and discusses the relationship of casein to total protein in milk, and states that the relationship between casein and fat appears to be linear. Azarme (42), in a study of the milk of twenty-seven cows maintained under comparatively constant conditions, and determining protein nitrogen by the Kjeldahl method, obtained a correlation of percentage total protein nitrogen to total yield of milk of $-0{\cdot}454$, while the correlation between yield of milk and amount of total protein nitrogen was $+0{\cdot}951$. He describes the secretion of the components of the nitrogen fraction of the milk during lactation. A regular and highly significant decrease in the percentage of total protein

nitrogen was observed from the beginning until the fourth week of lactation; from this date it rose gradually and then more steeply towards the end of the lactation. The yield of total protein nitrogen showed a constant parallelism with the lactation curve for the total yield of milk. Casein nitrogen was found to compose about 77% of the total protein nitrogen in the milk, and similar results were obtained. (Yield of milk to casein content, $r = -0{\cdot}318$. Yield of milk to yield of casein, $r = +0{\cdot}927$.) The albumin and globulin nitrogen is of the same general trend, though less marked. With regard to the possibility of nutrition affecting the yield of protein nitrogen, the author is of opinion that, if the amount of protein in the ration is diminished to such an extent as to exhaust the body protein, the result is not a milk poorer in protein, but a smaller quantity of milk richer in protein. He noted an increase in percentage of total nitrogen in the case of those cows which, when in season, declined in their total yield of milk. Owing to the exceptional conditions under which the experimental material was kept, it is probable that the average herd would show a somewhat greater variation.

Gaines & Overman (43) studied whether, provided the fat percentage of a cow were known, it would be possible to estimate the corresponding protein percentage. Samples were taken at 5-week intervals from the milk of individual cows in the University of Illinois herd. The elaboration of milk fat requires the elaboration of milk protein additional to that of fat-free milk secretion, and the writers conclude that any programme of altering the protein/calory ratio of the milk by selective breeding appears rather hopeless. Reder (44), in a study directed primarily to rancidity, analysed 290 samples for total protein, and finds a decrease in protein from the first to the second month, followed by an increase lasting to the seventh month, which is maintained till the end of the lactation. McCandlish (45), examining the protein supply for dairy cows, stated that, any feed rich in protein suddenly added to the ration in large quantities, results in a temporary increase in the percentage of fat, but that feeds high in protein had no specific or permanent influence on the fat content of milk. Morris (46) reviews the whole subject, and maintains that, if the protein of the ration is deficient either in quantity or quality, then that of the milk will be drawn from the body protein of the cow. If the difference cannot be made up, the milk yield will be reduced, and may become inadequate for the nutrition of the suckling young. In a study of the lecithin content of milk from three dairy cows, Horrall (47) found a fairly constant percentage in the fat after the fourth day of the lactation period. Udder infection caused an increase in this percentage. Azarme (48), on the material used in his previous work, discusses the question of errors involved in the estimation of the lactation yield of protein according to the intervals between sampling. A high degree of accuracy is obtained if samples are taken weekly during the first 6 and last 4 weeks of the lactation, the remainder being fortnightly. For practical purposes, however, a sufficient degree of accuracy will be obtained by fortnightly samples during the periods of rapid change and at intervals of 3 weeks during the remainder of the lactation: these results are valid only when the daily yields of milk are known; if the yield of milk is recorded at intervals, then the degree of error is increased.

Other constituents

According to Reder (44) there is not much variation in the lactose content between individual animals. There occurs a slight increase in the lactose content during the first twelve weeks of lactation, followed by a decrease during the succeeding 12-week period, after which it remains constant till about the 36th week, when a very gradual decrease sets in. Rancidity gives milk a lower chloride content. Azarme (42) states that decrease in the percentage of lactose is counterbalanced by an increase in the percentage of protein and ash in such a way that the percentage of solids-not-fat

remains constant. Sharp (49) reports an inverse relationship between lactose and ash constituents.

Reder (44) found a wide variation in the mean chloride content of the milk during a given lactation, individuals ranging from 0·091 to 0·136%. The general trend of chloride content during lactation is characterized by an initially high value which falls during the first four weeks, rising gradually again till about the 38th week, when the rate of increase becomes more pronounced, and continues till the end of the lactation. Jones & Davis (50) examined the milk of twenty Shorthorn and Guernsey cows as regards chloride and sodium. Inasmuch as the chloride content increases with advance in the period of lactation, an increase in the sodium content is to be expected. Figures are given for the breeds in this respect, but they are not statistically significant. Woodward *et al.* (38) report a progressive increase in the chloride content of the milk from the beginning to the end of the lactation period. Davies (51) found no great difference between the distributions of the chloride content of Shorthorn and Guernsey milk but Ayrshire contained roughly 10% and Friesian 40% more, but these figures are not conclusive. Environmental factors affecting chloride are discussed; it increases with a drop in solids-not-fat, and a change to spring grass lowers the content. There is a complementary relationship between chloride and lactose and the sodium content can be calculated from the chloride by an equation.

Van-Landingham *et al.* (52) report a lower content of inorganic phosphorus in the blood of lactating dairy cows during winter and early spring than during summer and autumn. In a study by Lunde & Closs (53) on the influence of feeding of seaweed on the iodine content of the milk, previous observations are confirmed, that the iodine content of the milk is directly affected by the quantity in the feed. It is noted that both the quantity of the milk and its fat content show negligible rises in seaweed-fed cows. 10% of the iodine content in the fodder was normally excreted in the milk. Most of the iodine was free in the milk, but 3·8% was linked to the fat and 6% to the protein.

Krauss *et al.* (54), irradiating Holstein and Jersey milks, report that the solids-not-fat fraction of Holstein milk may contain more material capable of being activated than that of Jersey milk. Stewart & Tocher (55) conclude that, while milk deficient in solids-not-fat can be improved in quality and quantity by adequate feeding, the predominating factor in its causation is heredity. Comparing A.I.V. fodder and artificially dried grass, Watson & Ferguson (31) found that the former depressed the percentage of solids-not-fat, whereas the latter appeared to increase it. McDowall (41) reviews at some length the subject of solids-not-fat. Reder (44) gives monthly changes in the *p*H content and studied lipase: all milk contains a small amount of lipolytically-active substance, rancidity making it more active.

Colour and flavour

Watson & Ferguson (31), Tucker *et al.* (56), Treichler *et al.* (57) and Tucker *et al.* (58), find that feed influences colour of butterfat, depending on breed, fat content of the milk and stage of lactation. Tucker *et al.* (56) found a definite association between high yellow colour and good flavour. Hening & Dahlberg (59) report flavour unaffected in a certain feeding experiment.

Vitamin content

A large number of papers confirm the preponderating influence of feed upon vitamin content of milk. Reviewing the subject, Turner (2) notes that B, being formed in the rumen by bacterial action, is not affected by the kind or quality of feed, and that the amount of C in the ration has little influence on its content in the milk. Vitamin G is identified with the yellow pigment of milk whey (lactoflavin). Treichler *et al.* (57)

confirm that the vitamin A content of milk is chiefly determined by feed, a result also obtained by Shaw *et al.* (60), by Hathaway *et al.* (61), and Copeland (62). Increase in carotene content of the butterfat is found by Hodgson *et al.* (63) not to be in proportion to the increase of carotene in the feed. The carotene in butterfat accounted for only a small proportion of the vitamin A activity. Whitnah & Riddell (64), in an examination of forty cows of four breeds, found that Jerseys had the highest vitamin C content, and that the higher values were significant; while the numbers were too small to allow of a definite conclusion, it appears that individuality and breed of cow do affect the vitamin C content. Rasmussen *et al.* (65) hold that the stage of lactation has a more definite effect on the vitamin C content. Kon & Henry (66) found no significant difference in vitamin D content between Guernsey and Shorthorn butterfats, while Wallis (67), in a paired feeding experiment with Jersey and Holstein cows, in which the food intake was controlled so that each animal received the same amount of vitamin D, states that, owing to the larger milk production of the Holstein, the total amount of vitamin D recovered in the milk was the same as in the Jersey cow. Knapp & Churchman (68) report the feeding of cacao shell as a source of increase in the vitamin D content of milk and butter. Studying the milk of six Holstein cows, Wallis & Olson (69) found a marked seasonal difference in vitamin D content, summer milk containing four times the amount of winter milk. According to Henry & Kon (70) the colostrum contains relatively more vitamin A and carotene than vitamin D.

Curd tension

McDowall *et al.* (71), with cows from the Massey Agricultural College herd, find that renneting time tends to rise with the progress of lactation, particularly in the case of Ayrshires. Breed differences exist in average renneting time (in decreasing order) in the Ayrshire, Friesian and Jersey. The longer renneting time is an indication of the softness of the curd. It was found that the interrelation between fat content and curd time is a breed character, there being no relationship in the Jersey milk, while in the Friesian and Ayrshire curd firmness increases with the fat content. Doan (72) has made a fairly complete review of the literature on this subject and deduces that soft curd milk is not a different kind of milk but one lower in solids and of high water content. There seems little doubt that cows carefully selected for the low tension character of their milk could be maintained as a herd.

Conformation

In a study by the Bureau of Dairy Industry, U.S. Department of Agriculture by Swett *et al.* (73), an interim report is made of a study of external body measurements as an index of production on 593 cows, with the co-operation of nineteen Agricultural Experiment Stations. Details are given of the measurements, and the results are presented by breeds, but no conclusions are drawn: certain post-mortem measurements are also given. Swett *et al.* (74) compare the milk and butterfat-producing ability of four Hereford with two Holstein cows, all maintained under normal dairy herd conditions: ante- and post-mortem measurements are given: full data of the yields and udder construction of the different animals are available, but most interesting of all are the post-mortem studies of the size and capacity of the udder. Since 1932 the American Jersey Cattle Club has made provision for the inspection and rating of Jersey cows solely on a basis of conformation, the inspections being made by approved judges, the cattle being rated in six classes from "Excellent" to "Poor". The results, reported by Copeland (75), indicate that there is some relation between the conformation of a cow and her producing ability, and that both good conformation and high production can be combined in the same animal, the qualities being not antagonistic to each other. The writer points out that it is a fallacy to suppose that, by breeding

solely for production, it will be possible to secure ideal breed type, or, by breeding solely for type to secure ideal production. Bartlett (76) tabulates records of type, size and production obtained from first prizewinners of different breeds at the London Dairy Show over a 10-year period. He finds a striking similarity between the body measurements of the inspection winners and the milking trial winners of the same breed, which makes it impossible to select any outstanding measurement as indicative of inspection or production features. Inspection winners were, on the average, 2 lb. heavier than milking trial winners, which were slightly larger as judged by height and length of body. There are interesting observations on the comparison of the different breeds, the outstanding feature of the Ayrshire being small chest measurements, while the Channel Island breeds possess the smallest bone in the foreleg. He notes that, with the exception of the Shorthorn, the breeds studied are probably larger at the present day than their remote ancestors. Mackintosh (77), gives an historical review of the relation of conformation as a means for selecting dairy cattle, and concludes that no point of conformation or other feature is of any value within a breed as an indication of the fat percentage in the milk of any cow. He holds that the escutcheon is no indicator, nor are the skin secretions. He maintains, that judging by inspection is of importance, and recommends competitions where from 30 to 50% of the total marks are awarded in respect of conformation. Zorn (78) gives post-mortem measurements of a high-yielding, long-lived East Friesian cow. Recently several claims have been made that the rate of growth of the calf may be taken as an indication of her ultimate milking capacity. From the growth records of the University of Nebraska Holstein herd (79), seventy-six females were selected for this study, using, as indices of growth, gain in weight, increase in height at withers and increase in chest girth. Weights and measurements are given in detail, with milk and fat production for the first lactation and for the lifetime average of lactations. While the animals were apparently normal as compared with standards established in the herd, no apparent correlation was observable for any of the three measurements with the recorded milk production. Kislovsky (80) made correlations between the measurable form of cows (and their indices) at different ages and their milk yield at the same period. Not only did the absolute value of the correlation vary but even its sign changed from + to −. Indices characteristic for early development were positively correlated with milk yield in young animals of from $1\frac{1}{2}$ to $2\frac{1}{2}$ years, whereas the same indices were negatively correlated at over $4\frac{1}{2}$ years. He concludes that a sound judgment of conformation can only be obtained if the living form is considered from its ontogenetic stage of development under a given environment, and that attention must be paid to its general constitution. See also the paper by Davydov and others (110) under "Progeny Test," which is in partial agreement with the conclusions of Kislovsky.

Efficiency of production and growth

Gaines (81) discusses the working maintenance and live weight relation of dairy cows, and concludes that, for practical utility, as well as a biological measure, he prefers the formula FCM/IW, in which FCM is milk energy yield for some suitable uniform period of the lactation, and IW (initial weight) is live weight of the cow at the start of the period. Gaines & Overman (43) discuss the protein content as it affects the energy value of milk. They maintain that the energy yield tends to be a simple multiple of the protein yield. Brody & Cunningham (82) find that the net energetic efficiency is of the order of 60%, and that the law of diminishing returns appears to apply to efficiency of milk production, as well as to growth. Working with the same data as the previous authors, Gaines (83) shows that, as regards these 369 cows of Jersey, Guernsey and Holstein breeds, milk energy yield per unit of live weight is

independent of live weight ($r = -0{\cdot}023$): gross efficiency increases with live weight ($r = +0{\cdot}345$). Then, since elimination of systematic errors in the feed record may reduce the latter correlation to zero (i.e. independent of live weight), a permissible deduction is that feed of working maintenance is proportional to live weight.

Headley (84), on the figures of Perkins (85), has constructed a nomograph chart for the easy calculation of Fat Corrected Milk when the yield of milk and percentage of fat is known. Brody & Ragsdale (86) find height at withers independent of environment. This is confirmed by Davis *et al.* (87) in Nebraska on data based on 10,921 pairs of measurements of females of all ages from birth in the four principal breeds of America: a formula is given for predicting the weight of a cow from her chest girth in relation to height at withers. Brody & Cunningham (88) find that gross energetic efficiency is not dependent physiologically on the body weight of the cow, but that the monetary profit is.

Krizenecky (89) has compared the efficiency formulae of Gaines and of Brody at five levels of production and three weights: Gaines' formula gives higher values, the difference increasing as production rises and weight falls. In another paper (90), studying the formula of Brody & Proctor dealing with food consumption in relation to fat-corrected milk production and live weight, he concludes that the tables may be used with safety for all breeds. For examination of live weight in relation to production, Krizenecky (91) studied 31,742 lactations, using mean values for weight classes in five testing stations: performance capacity remained more or less constant for all classes. Reviewing the whole subject of live weight and milk production, Krizenecky (92) defines the interrelation of weight and age for which discrimination must be made in the correction of records. Relation between weight and maintenance is not linear but according to 0·75 of the live weight, thus correcting earlier studies that assumed it was linear, and he concluded that while heavier cows are more economic, the relation between weight and efficiency is slight. Within weight classes the most important factor affecting economy is milk production: tables are given. Kleinberg (5) with 13,914 lactations over 7 years, gives figures for changes in live weight for age in Simmental, Pinzgau and Bernese cattle. Milk production showed increases of 420, 325 and 566 lb. respectively for each 100 lb. increase in weight: fat percentage showed a slight decrease with increasing weight in two breeds: fat yield increased with weight at the rate of 15, 12 and 27 lb. per 100 lb. increase in weight. The Bernese cows are more early-maturing and more affected by age than the others. Length of lactation was unaffected by age and correlations of milk production; percentage fat and total fat are independent of age. Gaines (93) has recently made a statistical examination of the nutrients required for lactation: it is possible that this may form the basis for genetic studies, but the writer of this review holds that, useful—if not indispensable—as these formulae are for nutritional studies, genetic research should be directed rather to the individual components of the lactation yield: once the modes of the inheritance of the components are understood, then there will be time to consider a universal figure for the lactation.

Black & Knapp (94) describe the relative growth of beef and dairy Shorthorn calves, and find that, to reach a certain weight, greater skeletal growth is necessary in the milking than in the Beef Shorthorn, the former growing more rapidly. A paper on the Nebraska State herd by Morgan & Davis (95) deals with the effect of pregnancy on the weight of calves and of their dams: no consistent breed or gestation relationship was discerned. Kiener (96) has produced a massive work dealing with Simmental cows with particular references to the hereditary characteristics of two families. The work is too detailed to quote here but should be examined by those interested in the measurements and colour of dairy cows in relation to production.

THE PROGENY TEST

Conklin (97) reviews the application of the progeny test to problems of livestock improvement, and refers to the waning enthusiasm for this method on the part of many breeders who have been disappointed in the practical application of the test. He agrees, however, that the proven sire is one of the major means for breed or herd improvement. Glover (98) discusses the use of progeny-tested bulls in different herds, and states, on observations made by Pennsylvania College of Agriculture, that, if a bull is good in one herd, he is usually equally good in other herds. The need for sire survey work is stressed by Ward (99), who gives an excellent example of the yields of fourteen daughters as compared to those of their dams, the number of lactations for each cow averaging about five: he believes that it is unwise to express the results of a survey in a single index of production for the sire: details are given of the New Zealand Sire Survey Scheme. The New Zealand Dairy Board (100) reports on the sire survey work which it conducts and publishes a table showing the results of the sire surveys with productions measured in pounds butterfat. The results are classified according to the production of the cows to which the bulls were mated, and show the regression to the mean, which has been recognized for several years as a feature of progeny testing.

Dams' production lb. fat	Number of bulls	Results of Progeny Test Daughters compared to dams		
		Improved	Maintained	Lowered
Below 320	9	7	1	1
320–359	42	11	20	11
360–399	38	9	11	18
Above 400	11	1	2	8

This survey probably shows the position as better than it really is since many herd owners refused to sanction a survey unless they were of opinion that their bull was a good one.

Goodale (101) examined the progeny test in so far as it could control the growth rate of mice, in which he was successful in increasing weight by 10 grammes, with little overlapping in weight, between the original mice and their descendants. He claims that the success which has attended this experiment is the result of consistent application of genotypic selection, and holds that the possibilities for the improvement of farm animals by the progeny test is much greater than is realized by breeders. He points out that natural selection is not wholly phenotypic since a larger proportion of the offspring of some matings survive than do those of other matings. The Mount Hope Index (referred to in previous reviews) forms the basis for a new Dairy Cattle Improvement Association in the United States. In the reports of the American Dairy Cattle Club (102, 103), details are given of the most recent method of application of this index, and lists of sires of five breeds in America. In a report on the results at Mount Hope Farm, Goodale (104) stresses the judging of a dairy bull by the whole of his progeny and illustrates how the proper application of the progeny test is a most effective method of breeding for improved performance.

Lush & Schultz (23) studied the progeny performance of 303 Holstein-Friesian sires in the Iowa Cow Testing Association. (For ease of description, they use the term "mates" to designate the dams of the daughters of the bulls, i.e. the cows to which the bulls were mated—a useful designation which it would be wise to adopt.) The mates have an average yield of 416 lb., and their daughters 430 lb. butterfat. The correlation between dam and daughter was +0·64, unusually high, but due to the fact that the averages of the dams have been correlated to the averages of the daughters, as distinct from the usual method of individual dam and individual

daughter. A negative correlation of −0·38 was obtained between the yields of the dams and the increase in the yields of their daughters: this is due to the well-known tendency for offspring to regress from a parent toward the mean of the race. This regression is partly because a cow's record is affected by other things than her breeding value, and cows with the highest records do not usually have as good a genetic constitution as their records would indicate, while cows with low records are not usually as poor as those records: also best cows are not necessarily mated to best bulls: since daughters obtain half their inheritance from each parent, they tend to be poorer than their dams when the dams are good, and vice versa. A positive correlation of +0·47 obtained between the yields of daughters and their increase is another way of expressing the same regression. The 14 lb. average increase in one generation cannot be considered to measure the true rate of genetic improvement since other possibilities affect this figure: records of the dams were probably more highly selected than those of the daughters; and increasing experience in testing leads to improved management of daughters. Lush & Schultz also studied the pedigrees of the bulls in relation to the productivity of the dam and grandams of each, and of the sire and grandsires, which were assessed by the progeny test of their daughters. Records of the dams and grandams indicate that intense selection is practised in deciding which dams are good enough to have their sons saved. Bulls with testing in their pedigrees were usually mated to cows of higher production than were other bulls. Considering one ancestor at a time, correlations were made between a bull's performance and the test of the ancestor: these were prevailingly positive, but small, and without statistical significance. The figures are interpreted as indicating that the relation between the bull's pedigree and his performance is slight; but it must be remembered that the ancestors were considered one at a time, whereas the purchaser of a bull combines the records of all to arrive at a weighted estimate of the desirability of the pedigree. The writers discuss the accuracy of the progeny test as an estimate of the true breeding value of a bull, and they conclude that pedigree promise is worth something in selecting a bull, but as a cold-blooded business proposition it should not be valued too highly.

Tufts(105) emphasizes the danger of estimating the value of a bull on the phenomenal production records of only a few daughters, and points out that the best daughter of a poor bull may be as good, or better than, the best daughter of a really good bull. He illustrates this from practical experience with the Ayrshire breed in the United States. Buchanan Smith & Robison(106) discuss the variations in the milk yields of the daughters of different bulls, using Fisher's squared difference method. Bulls are only compared with each other when their daughters have made records in the same herd, and at approximately the same time. They believe that more attention should be paid to the variance in daughters' yields than merely to their average yield, and that this is required if substantial progress is to be achieved by the use of the proven sire.

Dahlberg(107), discussing the question of sire indices, puts forward a compensated index. He quotes a case where an index was calculated on the equal parent principle, which showed that four bulls decreased the production of the daughters, but that, at the same time, the herd production increased. Johansson(9) prefers the Hansson-Yapp index, and believes that, if improvement is to be achieved, more emphasis must be laid on the progeny test. The same author(19) gives the result of the progeny tests of bulls used in a Swedish herd from 1921 to 1936: none increased the milking capacity of their daughters in comparison to the dams: he believes that the success of the herd is due not so much to constructive breeding as to rigid culling. Bosma(25) gives full details of the progeny test and "preferent" bulls in Holland. This Dutch work is also dealt with by Leroy(21). Willard & Quayle(108) report on proven sires in Wyoming.

Linčenko (109) reports records of fifteen years from the Ukrainian Institute. The first lactation represents a reliable basis for judging milk production, giving a high correlation with yield during the following five lactations. The productivity of a bull's daughters was estimated mainly by comparing their yield with that of the whole population. The dam/daughter comparison was found less satisfactory owing to the necessity for correcting for age and variation in environment. Results of testing six bulls are reported in detail. Davydov *et al.* (110) put forward the interesting suggestion that it might be possible to make an early evaluation of dairy sires by the birth weight of their calves. They report a marked correlation between the live weight of the cow and her milk yield ($0{\cdot}431 \pm 0{\cdot}105$), and also between the birth weight of a cow and her adult weight ($0{\cdot}404 \pm 2{\cdot}709$). Thus the theoretical correlation between the birth weight of a calf and her subsequent milk yield may be assumed to be of the range of $r = 0{\cdot}20$; the authors' empirical data vary from $r = 0{\cdot}088$ to $0{\cdot}363$. To ascertain the influence of the genotype of the sire on the birth weight of the calf, a detailed analysis has been made of non-genetic influences, and this has been determined by comparing the coefficient of correlation for the different groups studied. The results show that 51–80% of the observed phenotypic variability are due to genetic causes. The author advocates the use of correction factors in order to eliminate non-genetic variation of birth weight. Experimental tests of three bulls by the birth weight of their calves gave consistent results as compared with the usual method of proving a bull by the milk yield of his daughters.

Reproduction and wastage

Casida & Venzke (111) emphasize that shorter calving intervals are important if the highest average daily production is to be obtained from a dairy cow. A study of the breeding records of two herds indicates that, on the average, 60 or 70 days elapsed between calving and the first succeeding heat period. Clapp (112), reporting the Pabst Farms of Holsteins, found no significant correlation between butterfat yield and the length of interval to the first heat or other measurements of production in relation to breeding efficiency. Cows conceiving their second calf at one service, and those requiring more than one service did not differ significantly in production. As regards lifetime records, age had no effect on the interval of first heat. When intervals to first heat were compared for tested cows, non-tested cows and nurse cows, striking differences were found. The interval for cows on test was 69·4 days, for nurse cows 71·8 days, while for cows that were neither nurse nor on test, 46·4 days. The author states that the frequency of suckling or handling of the teats in milking is the main cause of the difference in the length of interval to first heat between the cows milked twice a day and those milked four times a day or used as nurse cows. Mead (113) describes a diagrammatic method of showing at a glance the reproductive history of an entire herd.

In a study of the breeding efficiency of twenty proven sires used in the herds maintained by the Bureau of Dairy Industry, Dawson (114) found that seventeen out of the twenty examined averaged $4\frac{1}{2}$ years' fertile service in the herd after they were five years of age. The average number of services per conception was 2·49. Considerable variation was found in the fertility of the different bulls, varying from 21 to 71%. He concludes that, because of the extreme and inconsistent variation in fertility exhibited by individual sires, it is apparent that averages are of little value for application to individual bulls. Mullen (115) reports on wastage in Lancashire. Animals with low milk yield accounted for 14% of the total disposals. Two herds in Hertfordshire are compared by Hunter Smith (116). The poor milking qualities of home-bred heifers were one of the largest sources of loss. Examining cows culled from tested herds in Iowa, Seath and Lush (117) report that 29% left these herds each

year. Tables of life expectancy are given for these cows. Records from the Iowa State College dairy herd by Ingels & Cannon (118) for 29 years show that only 55% of all heifers born entered the dairy herd. If this figure is typical, it shows that a very meagre margin is left for replacement on which the dairy herd can be maintained. Mackintosh (77) emphasizes the importance of conformation as an index of the lifetime producing ability of the cow. Certainly, the rise of the 50-ton milk producers and 100,000-lb. clubs emphasizes this quality. Battha (119) emphasizes "life efficiency" and claims that young cows suffer through attempts to obtain record yields. Schaper (120) claims that constitutional diseases cannot be regarded as a result of high yields. Engeler (121) contributes to this discussion a balanced statement of the pros and cons. Copeland (27) makes a distinctive contribution to this subject; he holds that lifetime productions are influenced by longevity, opportunity and good fortune. The inheritance of high milk-producing ability, longevity, breeding efficiency, disease resistance and strength of udder attachments, all contribute to the end product of profitable lifetime production. Yet these are separate problems in themselves, and he maintains that selection of the highest record of an animal is the best index as to that animal's actual inherited production capacity under normal conditions.

Examining factors that affect the resistance of cows to mastitis, a group of workers in the U.S. Department of Agriculture, Burkey *et al.* (122), found that the germicidal property of the milk differs in individual cows, and that by means of leucocytes the cow has significant defensive agencies against infection. Ward (123) finds, amongst cows in the same herd, very varying degrees of resistance to mastitis. The work is not conclusive, but is extremely suggestive. He gives the following figures:

	Daughters			
	No mastitis		Mastitis	
Dams	No.	%	No.	%
No mastitis	176	76	54	24
Medium infection	54	50	54	50
Heavy infection	111	39	171	61

He also studied the herds from the point of view of sire-daughter and adduces the fact supporting the assumption that the diathesis to at least certain forms of mastitis has a genetic basis. For instance, one sire left practically all his twenty-five daughters susceptible to this disease.

It has long been suspected that susceptibility to milk fever might be inherited. Metzger & Morrison (124) report on 218 cows of Jersey, Holstein and Guernsey breeds maintained and housed as one herd at the Kentucky Agricultural Experiment Station. No milk fever occurred with the first calving, but the data show that cows become susceptible with the second, and this increases to the fifth or sixth, and that many of the cows showed a tendency to have more than one case, this being particularly marked in the Jerseys. A study of the pedigree failed to give evidence to enable one to say that a tendency to have milk fever is inherited. The authors point out that, as one breed showed an increased susceptibility, and as milk fever occurs most often in high-producing cows, there is a possibility of an inherited tendency. The occurrence of milk fever may also depend on the month of calving and the general climate of the year.

Breed and herd construction—inbreeding

Bosma (25) gives a good review of work accomplished with the Dutch Black-and-White cattle during the past fifteen years. The cattle registered in the general Dutch Herd Book are similarly dealt with. No significant difference was found in the milk

yield in respect of either breed between fully pedigreed cows and those on the "grading up" register. The author notes that the Dutch breeders tend to select for high fat content, and that there is not much evidence that the leading breeders consciously select for high milk production. So far as milk yield is concerned, animals which have an important influence on the breed may not themselves come up to a high standard of excellence. Abts (11) reviews the development of the Black Pied Lowland cattle of Germany during the past twenty years. Engeler (125) describes the growth of herd books in Switzerland.

The history of the Ayrshire breed is discussed by Buchanan Smith (126) in what is a breed survey rather than a genetic study. As well as tracing the history of the breed, this paper covers its distribution throughout the world. The *Canadian Ayrshire Record* has published volume 2, which is an excellent example of how some progressive breeding societies are tackling the problem of combining pedigree with performance in their records. Lists are given of high-producing cows and progeny-tested bulls. Wilson (127) describes recording methods in Tasmania. Johansson (19) has made a study of the production records of one herd in Swedish Red-and-White cows, and most of his conclusions are discussed elsewhere, but, amongst others, he reached an indefinite conclusion on the relative importance of environment and heredity. Plum (128) describes the breeding methods employed in a Danish herd tracing back to 1892, and calculates the coefficients of inbreeding which, at one period, rose to 8%, but thereafter dropped through the use of an outcross bull. The inbred animals hardly did so well at the livestock shows and there appears to be no significant difference in the transmitting ability of the inbred and non-inbred bulls as regards the uniformity of butterfat production in their daughters. The degree of inbreeding, however, was not intense, and there was a big difference in the blood lines of the inbred and non-inbred strains. Lush *et al.* (129) have written a genetic history of Holstein-Friesian cattle in the United States, while Yoder & Lush (130) have accomplished the same for the Brown Swiss of that country. Neither study shows appreciable inbreeding and the high producers do not differ in this respect from the average of their breed. A revised edition of *British Breeds of Livestock* (131) has been published by the Ministry of Agriculture: it includes brief descriptions of all the dairy and dual-purpose breeds.

Tropical

Wright (132) has a comprehensive report on the question of the improvement of dairy cattle in India. No substantial improvement in the way of breeding is possible until the cattle can be better fed and until there is adequate control of disease. The question is discussed of the issue of bulls by the Government as a means for improving the quality of the stock throughout the country. The writer advocates that this policy should be pursued with greater diligence, as also the castration of inferior males. The average yield of milk of the Indian dairy cow is only slightly over 600 lb. per year, representing a daily yield of about half a gallon. Crossing with European breeds has been successful when restricted to the first cross, particularly at the Military Dairy Farms, but further breeding with imported blood is not particularly promising. Backcrossing to native cattle improves constitution, but reduces yield. Deterioration in the constitution of adult stock appears to be due not so much to crossbreeding as to the effect of high yield under the rigorous climatic conditions of India: there are indications of increased susceptibility to disease and loss of constitution, even among Indian dairy cattle, when their milking performances improve. A report on the Government Sahiwal herd at Pusa, which covers a period from 1913 to 1937 has been published (133). The figures contained are interesting. The Imperial Council of Agricultural Research have recently (134) published an illustrated survey of

some of the important breeds of cattle in India, with excellent illustrations. Representative milk yields are given. To this Olver contributes a critical survey of the origins of Indian cattle, which he roughly classifies into five basic types; he describes representatives of the principal breeds of each type. Reporting that systematic investigation carried out by the Animal Husbandry Bureau of the Imperial Council of Agricultural Research in India has shown that, by careful selection and proper feeding and management, herds of pure Indian cattle have been produced in 25 years, Olver (135) states that they can more than hold their own in India with European cattle and the best Indian buffaloes. Some of these herds already exceed the milk yield of commercial herds in Europe and America: he states that even with the best of care, cattle of European origin tend to degenerate in India. In this paper he describes how the improvement of dairy cattle in India should proceed and states that more milk should be bred into the Indian breeds of work cattle.

Watson (136) describes the acclimatization of Ayrshire, Jersey and Holstein cattle in Hong Kong. Permanent sterility is more common in imported animals than in those bred locally. Stuchbery (137) describes herd testing in Fiji. The breeding efficiency of grade Ayrshire cows in the Philippines was found by Fernandez (138) to be about 67%, but varies according to conditions: Sussex × Nellore cows averaged 50·6%. Williams (139) reports observations for 14 years on a pure bred Holstein-Friesian herd maintained in a subtropical part of the U.S.A. Specially noted are sexual weakness in bulls, as well as cows, fertility being conditioned by the weather: when breeding efficiency was high, the sex ratio was low, and vice versa.

Kothavala & Sunawala (140) studied the number, size and shape of the fat globules in seven dairy and four draft breeds of Indian cattle, as well as three breeds of buffalo. In the milk of the buffalo the fat globules were large and the number low as compared to cow's milk. Fat globules are more numerous and more uniform in the milk of the draft than in the dairy breeds.

Other references to dairy cattle problems in the Tropics are to be found in the reports of Government departments concerned. Particular reference might, however, be made to Bihar & Orissa (141), the *Annual Report* of the Imperial Dairy Expert in India (142), Jamaica (143) (where the milk production averages by breeds and lactation periods are given), and Palestine (144) (where the Brown Swiss has acclimatized well).

More attention requires to be given to the subject of the water buffalo. In addition to the paper by Kothavala & Sunawala (140), mentioned above, is a book by Pissarewsky (145): it describes the distribution of these animals in different parts of the world, their management, the conformation, signs of milk, etc.: figures are given on breed differences in yield and how it is affected by age, season, nutrition and management. Further information on this subject is to be found in the Madras *Report* (146), and in that of Olver (135).

Artificial insemination

Considerable work has recently been accomplished in the use of artificial insemination as a means for the improvement of livestock. Few results, other than Russian, are to hand so far as dairy cattle are concerned; the following may be noted: Cole (147) adjusts the technique to ordinary dairy herds. The large-scale work of the co-operative breeding society on the Island of Samsø in Denmark is described by Perry (148): during the first year of the operation of the scheme, 1200 cows were artificially inseminated with an average number of inseminations per conception of 1·3 as against 1·8 by the natural method: only two bulls were used. The first "international" calf born in Essex and sired by a bull in Holland is described in the *Farmer & Stockbreeder* (149). Details of this exchange of bull semen between England and Holland are further discussed by Edwards, Walton & Siebenga (150), who state that for transport the sample should be as dense as possible, preferably not less than

1000 million sperm per c.c. Bartlett (151) reports results at Reading in a herd affected by contagious abortion; 253 normal matings gave 38% fertility, while fifty-seven services by artificial insemination gave 32% effectiveness. Anderson (152) has described its application to tropical conditions in Kenya, where it is being carried out with considerable success and is also of value as a means for combating Trichomonas infection.

The New Zealand Dairy Board (100) have been carrying out artificial insemination, and stress the importance of accurate sire surveys in order that the maximum advantage may be derived from this technique.

Cole & Winters (153) inseminated 121 dairy cows in Minnesota with a mating ratio of 1 : 1·77 for cows definitely pregnant, four failing to conceive. They claim that it is not necessary to employ the services of a veterinary surgeon and that the successful insemination of 1000 or more cows per sire per annum is a definite possibility.

According to Pincus (154) a million cows were inseminated in Russia in 1937 and he estimates the figure at 1,500,000 for 1938.

Dawson (155), dealing with the breeding efficiency of proved (aged) sires, shows that twenty studied in relation to 3585 services gave a ratio of 2·49 services per conception (based on fertile cows). There was considerable variation in fertility and one bull is reported as fertile at the age of 17 years. He deals with various factors that affect the fertility of aged bulls.

Based on records of the dairy herd of the University of Nebraska (156) from 1896 to 1934, covering five breeds and 2090 cows, young bulls under two years show the smallest number of services per conception, though from 2 to 8 years of age the number of services required varies little. Virgin heifers require more services per conception than any age-group of cows up to 10 years. Bulls 3 years of age and older were not efficient when mated with females under 2 years of age. Over all, the average number of services required per conception was 2·21, based on 1375 conceptions. 730 calves were males and 628 females, with thirty sets of twins and thirty-seven of unknown sex. The sex ratio was 53·75% males and 46·25% females. The average percentage of abortion was 12·58. From May to October 2·28 services were required per conception, while from November to April the figure was 2·14 in respect of bulls of all ages mated to cows of all ages. The monthly variation in number of services per conception varied from 1·94 in December to 2·86 in September. With the exception of November, all months showed more males than females in the sex ratio.

A book by Espe (157) on secretion of milk gives an interesting review, principally of American work. Somewhat too concisely, perhaps, it summarizes the genetic aspects and approaches the whole subject from a different standpoint from this review. The works of Gowen, Turner and Gifford are largely quoted, and a section has been revised by Lush. Another publication well worth consulting is the annual contribution by Mackintosh (158) to the Royal Agricultural Society of England. In his last he includes a critical survey of systems of milk recording.

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